

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICATION FOR PATENT OF:

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FOR:

**NON-ENDOGENOUS, CONSTITUTIVELY  
ACTIVATED HUMAN G PROTEIN-  
COUPLED RECEPTORS**

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**NON-ENDOGENOUS, CONSTITUTIVELY ACTIVATED  
HUMAN G PROTEIN-COUPLED RECEPTORS**

This patent application is a continuation-in-part of, and claims priority from, U.S. Serial Number 09/170,496, filed with the United States Patent and Trademark Office on

5    October 13, 1998. This application also claims the benefit of priority from the following provisional applications, all filed via U.S. Express Mail with the United States Patent and Trademark Office on the indicated dates: U.S. Provisional Number 60/110,060, filed November 27, 1998; U.S. Provisional Number 60/120,416, filed February 16, 1999; U.S. Provisional Number 60/121,852, filed February 26, 1999 claiming benefit of U.S.

10    Provisional Number 60/109,213, filed November 20, 1998; U.S. Provisional Number 60/123,944, filed March 12, 1999; U.S. Provisional Number 60/123,945, filed March 12, 1999; U.S. Provisional Number 60/123,948, filed March 12, 1999; U.S. Provisional Number 60/123,951, filed March 12, 1999; U.S. Provisional Number 60/123,946, filed March 12, 1999; U.S. Provisional Number 60/123,949, filed March 12, 1999; U.S.

15    Provisional Number 60/152,524, filed September 3, 1999, claiming benefit of U.S. Provisional Number 60/151,114, filed August 27, 1999 and U.S. Provisional Number 60/108,029, filed November 12, 1998; U.S. Provisional Number 60/136,436, filed May 28,

1999; U.S. Provisional Number 60/136,439, filed May 28, 1999; U.S. Provisional Number 60/136,567, filed May 28, 1999; U.S. Provisional Number 60/137,127, filed May 28, 1999; U.S. Provisional Number 60/137,131, filed May 28, 1999; U.S. Provisional Number 60/141,448, filed June 29, 1999 claiming benefit of U.S. Provisional Number 60/136,437, filed May 28, 1999; U.S. Provisional Number 60/156,633, filed September 29, 1999; U.S. Provisional Number 60/156,555, filed September 29, 1999; U.S. Provisional Number 60/156,634, filed September 29, 1999; U.S. Provisional Number \_\_\_\_ (Arena Pharmaceuticals, Inc. docket number: CHN10-1), filed September 29, 1999; U.S. Provisional Number \_\_\_\_ (Arena Pharmaceuticals, Inc. docket number: RUP6-1), filed October 1, 1999; U.S. Provisional Number \_\_\_\_ (Arena Pharmaceuticals, Inc. docket number: RUP7-1), filed October 1, 1999; U.S. Provisional Number \_\_\_\_ (Arena Pharmaceuticals, Inc. docket number: CHN6-1), filed October 1, 1999; U.S. Provisional Number \_\_\_\_ (Arena Pharmaceuticals, Inc. docket number: RUP5-1), filed October 1, 1999; and U.S. Provisional Number \_\_\_\_ (Arena Pharmaceuticals, Inc. docket number: CHN9-1), filed October 1, 1999. This application is also related to co-pending U.S. Serial Number \_\_\_\_ (Woodcock, Washburn, Kurtz, Makiewicz & Norris, LLP docket number AREN-0050), filed on October 12, 1999 (via U.S. Express Mail) and U.S. Serial Number 09/364,425, filed on July 30, 1999, both incorporated herein by reference. Each of the foregoing applications are incorporated by reference herein in their entirety.

## FIELD OF THE INVENTION

The invention disclosed in this patent document relates to transmembrane receptors, and more particularly to human G protein-coupled receptors, and specifically to

GPCRs that have been altered to establish or enhance constitutive activity of the receptor. Preferably, the altered GPCRs are used for the direct identification of candidate compounds as receptor agonists, inverse agonists or partial agonists having potential applicability as therapeutic agents.

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## BACKGROUND OF THE INVENTION

Although a number of receptor classes exist in humans, by far the most abundant and therapeutically relevant is represented by the G protein-coupled receptor (GPCR or GPCRs) class. It is estimated that there are some 100,000 genes within the human genome, and of these, approximately 2%, or 2,000 genes, are estimated to code for GPCRs. Receptors, including GPCRs, for which the endogenous ligand has been identified are referred to as "known" receptors, while receptors for which the endogenous ligand has not been identified are referred to as "orphan" receptors. GPCRs represent an important area for the development of pharmaceutical products: from approximately 20 of the 100 known GPCRs, 60% of all prescription pharmaceuticals have been developed.

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GPCRs share a common structural motif. All these receptors have seven sequences of between 22 to 24 hydrophobic amino acids that form seven alpha helices, each of which spans the membrane (each span is identified by number, *i.e.*, transmembrane-1 (TM-1), transmebrane-2 (TM-2), etc.). The transmembrane helices are joined by strands of amino acids between transmembrane-2 and transmembrane-3, transmembrane-4 and transmembrane-5, and transmembrane-6 and transmembrane-7 on the exterior, or "extracellular" side, of the cell membrane (these are referred to as "extracellular" regions 1, 2 and 3 (EC-1, EC-2 and EC-3), respectively). The transmembrane helices are also joined by strands of amino acids between transmembrane-1 and transmembrane-2, transmembrane-3 and transmembrane-4, and

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transmembrane-5 and transmembrane-6 on the interior, or "intracellular" side, of the cell membrane (these are referred to as "intracellular" regions 1, 2 and 3 (IC-1, IC-2 and IC-3), respectively). The "carboxy" ("C") terminus of the receptor lies in the intracellular space within the cell, and the "amino" ("N") terminus of the receptor lies in the extracellular space  
5 outside of the cell.

Generally, when an endogenous ligand binds with the receptor (often referred to as "activation" of the receptor), there is a change in the conformation of the intracellular region that allows for coupling between the intracellular region and an intracellular "G-protein." It has been reported that GPCRs are "promiscuous" with respect to G proteins, *i.e.*,  
10 that a GPCR can interact with more than one G protein. *See*, Kenakin, T., 43 *Life Sciences* 1095 (1988). Although other G proteins exist, currently, Gq, Gs, Gi, Gz and Go are G proteins that have been identified. Endogenous ligand-activated GPCR coupling with the G-protein begins a signaling cascade process (referred to as "signal transduction"). Under normal conditions, signal transduction ultimately results in cellular activation or cellular inhibition.  
15 It is thought that the IC-3 loop as well as the carboxy terminus of the receptor interact with the G protein.

Under physiological conditions, GPCRs exist in the cell membrane in equilibrium between two different conformations: an "inactive" state and an "active" state. A receptor in an inactive state is unable to link to the intracellular signaling transduction  
20 pathway to produce a biological response. Changing the receptor conformation to the active state allows linkage to the transduction pathway (via the G-protein) and produces a biological response.

A receptor may be stabilized in an active state by an endogenous ligand or a

compound such as a drug. Recent discoveries, including but not exclusively limited to modifications to the amino acid sequence of the receptor, provide means other than endogenous ligands or drugs to promote and stabilize the receptor in the active state conformation. These means effectively stabilize the receptor in an active state by  
5 simulating the effect of an endogenous ligand binding to the receptor. Stabilization by such ligand-independent means is termed "constitutive receptor activation."

### SUMMARY OF THE INVENTION

Disclosed herein are non-endogenous versions of endogenous, human GPCRs and uses thereof.

### BRIEF DESCRIPTION OF THE DRAWINGS

10 **Figure 1** is a representation of 8XCRE-Luc reporter plasmid (*see*, Example 4(c)3.)

**Figures 2A and 2B** are graphic representations of the results of ATP and ADP binding to endogenous TDAG8 (2A) and comparisons in serum and serum free media (2B).

15 **Figure 3** is a graphic representation of the comparative signaling results of CMV versus the GPCR Fusion Protein H9(F236K):Gsa.

### DETAILED DESCRIPTION

The scientific literature that has evolved around receptors has adopted a number of terms to refer to ligands having various effects on receptors. For clarity and  
20 consistency, the following definitions will be used throughout this patent document. To the extent that these definitions conflict with other definitions for these terms, the following definitions shall control:

**AGONISTS** shall mean materials (*e.g.*, ligands, candidate compounds) that

activate the intracellular response when they bind to the receptor, or enhance GTP binding to membranes.

**AMINO ACID ABBREVIATIONS** used herein are set out in Table A:

TABLE A		
5	ALANINE	ALA
	ARGININE	ARG
	ASPARAGINE	ASN
	ASPARTIC ACID	ASP
	CYSTEINE	CYS
10	GLUTAMIC ACID	GLU
	GLUTAMINE	GLN
	GLYCINE	GLY
	HISTIDINE	HIS
	ISOLEUCINE	ILE
15	LEUCINE	LEU
	LYSINE	LYS
	METHIONINE	MET
	PHENYLALANINE	PHE
	PROLINE	PRO
20	SERINE	SER
	THREONINE	THR
	TRYPTOPHAN	TRP
	TYROSINE	TYR
	VALINE	VAL

25 **PARTIAL AGONISTS** shall mean materials (*e.g.*, ligands, candidate compounds) that activate the intracellular response when they bind to the receptor to a lesser degree/extent than do agonists, or enhance GTP binding to membranes to a lesser degree/extent than do agonists.

**ANTAGONIST** shall mean materials (*e.g.*, ligands, candidate compounds) that  
 30 competitively bind to the receptor at the same site as the agonists but which do not activate the intracellular response initiated by the active form of the receptor, and can thereby inhibit the intracellular responses by agonists or partial agonists. **ANTAGONISTS** do not diminish the baseline intracellular response in the absence of an agonist or partial agonist.

**CANDIDATE COMPOUND** shall mean a molecule (for example, and not limitation,

a chemical compound) that is amenable to a screening technique. Preferably, the phrase "candidate compound" does not include compounds which were publicly known to be compounds selected from the group consisting of inverse agonist, agonist or antagonist to a receptor, as previously determined by an indirect identification process ("indirectly identified  
5 compound"); more preferably, not including an indirectly identified compound which has previously been determined to have therapeutic efficacy in at least one mammal; and, most preferably, not including an indirectly identified compound which has previously been determined to have therapeutic utility in humans.

**COMPOSITION** means a material comprising at least one component; a  
10 "pharmaceutical composition" is an example of a composition.

**COMPOUND EFFICACY** shall mean a measurement of the ability of a compound to inhibit or stimulate receptor functionality, as opposed to receptor binding affinity. Exemplary means of detecting compound efficacy are disclosed in the Example section of this patent document.

15 **CODON** shall mean a grouping of three nucleotides (or equivalents to nucleotides) which generally comprise a nucleoside (adenosine (A), guanosine (G), cytidine (C), uridine (U) and thymidine (T)) coupled to a phosphate group and which, when translated, encodes an amino acid.

**CONSTITUTIVELY ACTIVATED RECEPTOR** shall mean a receptor subject to  
20 constitutive receptor activation. A constitutively activated receptor can be endogenous or non-endogenous.

**CONSTITUTIVE RECEPTOR ACTIVATION** shall mean stabilization of a receptor in the active state by means other than binding of the receptor with its endogenous



ligand or a chemical equivalent thereof.

**CONTACT** or **CONTACTING** shall mean bringing at least two moieties together, whether in an in vitro system or an in vivo system.

**DIRECTLY IDENTIFYING** or **DIRECTLY IDENTIFIED**, in relationship to the  
5 phrase "candidate compound", shall mean the screening of a candidate compound against a constitutively activated receptor, preferably a constitutively activated orphan receptor, and most preferably against a constitutively activated G protein-coupled cell surface orphan receptor, and assessing the compound efficacy of such compound. This phrase is, under no circumstances, to be interpreted or understood to be encompassed by or to encompass the  
10 phrase "indirectly identifying" or "indirectly identified."

**ENDOGENOUS** shall mean a material that a mammal naturally produces. **ENDOGENOUS** in reference to, for example and not limitation, the term "receptor," shall mean that which is naturally produced by a mammal (for example, and not limitation, a human) or a virus. By contrast, the term **NON-ENDOGENOUS** in this context shall mean  
15 that which is not naturally produced by a mammal (for example, and not limitation, a human) or a virus. For example, and not limitation, a receptor which is not constitutively active in its endogenous form, but when manipulated becomes constitutively active, is most preferably referred to herein as a "non-endogenous, constitutively activated receptor." Both terms can be utilized to describe both "in vivo" and "in vitro" systems. For example, and not limitation,  
20 in a screening approach, the endogenous or non-endogenous receptor may be in reference to an in vitro screening system. As a further example and not limitation, where the genome of a mammal has been manipulated to include a non-endogenous constitutively activated receptor, screening of a candidate compound by means of an in vivo system is viable.

**G PROTEIN COUPLED RECEPTOR FUSION PROTEIN and GPCR FUSION**

**PROTEIN**, in the context of the invention disclosed herein, each mean a non-endogenous protein comprising an endogenous, constitutively activate GPCR or a non-endogenous, constitutively activated GPCR fused to at least one G protein, most preferably the alpha ( $\alpha$ ) subunit of such G protein (this being the subunit that binds GTP), with the G protein preferably being of the same type as the G protein that naturally couples with endogenous orphan GPCR. For example, and not limitation, in an endogenous state, if the G protein "Gsa" is the predominate G protein that couples with the GPCR, a GPCR Fusion Protein based upon the specific GPCR would be a non-endogenous protein comprising the GPCR fused to Gsa; in some circumstances, as will be set forth below, a non-predominant G protein can be fused to the GPCR. The G protein can be fused directly to the c-terminus of the constitutively active GPCR or there may be spacers between the two.

**HOST CELL** shall mean a cell capable of having a Plasmid and/or Vector incorporated therein. In the case of a prokaryotic Host Cell, a Plasmid is typically replicated as a autonomous molecule as the Host Cell replicates (generally, the Plasmid is thereafter isolated for introduction into a eukaryotic Host Cell); in the case of a eukaryotic Host Cell, a Plasmid is integrated into the cellular DNA of the Host Cell such that when the eukaryotic Host Cell replicates, the Plasmid replicates. Preferably, for the purposes of the invention disclosed herein, the Host Cell is eukaryotic, more preferably, mammalian, and most preferably selected from the group consisting of 293, 293T and COS-7 cells.

**INDIRECTLY IDENTIFYING** or **INDIRECTLY IDENTIFIED** means the traditional approach to the drug discovery process involving identification of an endogenous ligand specific for an endogenous receptor, screening of candidate compounds against the

receptor for determination of those which interfere and/or compete with the ligand-receptor interaction, and assessing the efficacy of the compound for affecting at least one second messenger pathway associated with the activated receptor.

**INHIBIT** or **INHIBITING**, in relationship to the term "response" shall mean that a  
5 response is decreased or prevented in the presence of a compound as opposed to in the absence of the compound.

**INVERSE AGONISTS** shall mean materials (*e.g.*, ligand, candidate compound) which bind to either the endogenous form of the receptor or to the constitutively activated form of the receptor, and which inhibit the baseline intracellular response initiated by the  
10 active form of the receptor below the normal base level of activity which is observed in the absence of agonists or partial agonists, or decrease GTP binding to membranes. Preferably, the baseline intracellular response is inhibited in the presence of the inverse agonist by at least 30%, more preferably by at least 50%, and most preferably by at least 75%, as compared with the baseline response in the absence of the inverse agonist.

**KNOWN RECEPTOR** shall mean an endogenous receptor for which the endogenous  
15 ligand specific for that receptor has been identified.

**LIGAND** shall mean an endogenous, naturally occurring molecule specific for an endogenous, naturally occurring receptor.

**MUTANT** or **MUTATION** in reference to an endogenous receptor's nucleic acid  
20 and/or amino acid sequence shall mean a specified change or changes to such endogenous sequences such that a mutated form of an endogenous, non-constitutively activated receptor evidences constitutive activation of the receptor. In terms of equivalents to specific sequences, a subsequent mutated form of a human receptor is considered to be equivalent to

a first mutation of the human receptor if (a) the level of constitutive activation of the subsequent mutated form of a human receptor is substantially the same as that evidenced by the first mutation of the receptor; and (b) the percent sequence (amino acid and/or nucleic acid) homology between the subsequent mutated form of the receptor and the first mutation  
5 of the receptor is at least about 80%, more preferably at least about 90% and most preferably at least 95%. Ideally, and owing to the fact that the most preferred cassettes disclosed herein for achieving constitutive activation includes a single amino acid and/or codon change between the endogenous and the non-endogenous forms of the GPCR, the percent sequence homology should be at least 98%.

10       **NON-ORPHAN RECEPTOR** shall mean an endogenous naturally occurring molecule specific for an endogenous naturally occurring ligand wherein the binding of a ligand to a receptor activates an intracellular signaling pathway.

**ORPHAN RECEPTOR** shall mean an endogenous receptor for which the endogenous ligand specific for that receptor has not been identified or is not known.

15       **PHARMACEUTICAL COMPOSITION** shall mean a composition comprising at least one active ingredient, whereby the composition is amenable to investigation for a specified, efficacious outcome in a mammal (for example, and not limitation, a human). Those of ordinary skill in the art will understand and appreciate the techniques appropriate for determining whether an active ingredient has a desired efficacious outcome based upon the  
20 needs of the artisan.

**PLASMID** shall mean the combination of a Vector and cDNA. Generally, a Plasmid is introduced into a Host Cell for the purposes of replication and/or expression of the cDNA as a protein.

**STIMULATE** or **STIMULATING**, in relationship to the term "response" shall mean that a response is increased in the presence of a compound as opposed to in the absence of the compound.

**VECTOR** in reference to cDNA shall mean a circular DNA capable of incorporating  
5 at least one cDNA and capable of incorporation into a Host Cell.

The order of the following sections is set forth for presentational efficiency and is not intended, nor should be construed, as a limitation on the disclosure or the claims to follow.

**A. Introduction**

The traditional study of receptors has always proceeded from the a priori assumption  
10 (historically based) that the endogenous ligand must first be identified before discovery could proceed to find antagonists and other molecules that could affect the receptor. Even in cases where an antagonist might have been known first, the search immediately extended to looking for the endogenous ligand. This mode of thinking has persisted in receptor research even after the discovery of constitutively activated receptors. What has not been heretofore recognized  
15 is that it is the active state of the receptor that is most useful for discovering agonists, partial agonists, and inverse agonists of the receptor. For those diseases which result from an overly active receptor or an under-active receptor, what is desired in a therapeutic drug is a compound which acts to diminish the active state of a receptor or enhance the activity of the receptor, respectively, not necessarily a drug which is an antagonist to the endogenous ligand.  
20 This is because a compound that reduces or enhances the activity of the active receptor state need not bind at the same site as the endogenous ligand. Thus, as taught by a method of this invention, any search for therapeutic compounds should start by screening compounds against the ligand-independent active state.

**B. Identification of Human GPCRs**

The efforts of the Human Genome project has led to the identification of a plethora of information regarding nucleic acid sequences located within the human genome; it has been the case in this endeavor that genetic sequence information has been made available without an understanding or recognition as to whether or not any particular genomic sequence does or may contain open-reading frame information that translate human proteins. Several methods of identifying nucleic acid sequences within the human genome are within the purview of those having ordinary skill in the art. For example, and not limitation, a variety of human GPCRs, disclosed herein, were discovered by reviewing the GenBank™ database, while other GPCRs were discovered by utilizing a nucleic acid sequence of a GPCR, previously sequenced, to conduct a BLAST™ search of the EST database. Table B, below, lists several endogenous GPCRs that we have discovered, along with a GPCR's respective homologous receptor.

**TABLE B**

	Disclosed Human Orphan GPCRs	Accession Number Identified	Open Reading Frame (Base Pairs)	Per Cent Homology To Designated GPCR	Reference To Homologous GPCR (Accession No.)
15					
	hARE-3	AL033379	1,260 bp	52.3% LPA-R	U92642
20	hARE-4	AC006087	1,119 bp	36% P2Y5	AF000546
	hARE-5	AC006255	1,104 bp	32% <i>Oryzias latipes</i>	D43633
	hGPR27	AA775870	1,128 bp		
	hARE-1	AI090920	999 bp	43% KIAA0001	D13626
	hARE-2	AA359504	1,122 bp	53% GPR27	
25	hPPR1	H67224	1,053 bp	39% EBI1	L31581
	hG2A	AA754702	1,113 bp	31% GPR4	L36148

	<b>hRUP3</b>	AL035423	1,005 bp	30% <i>Drosophila melanogaster</i>	2133653
	<b>hRUP4</b>	AI307658	1,296 bp	32% pNPGPR 28% and 29 % <i>Zebra fish</i> Ya and Yb, respectively	NP_004876 AAC41276 and AAB94616
	<b>hRUP5</b>	AC005849	1,413 bp	25% DEZ	Q99788
	<b>hRUP6</b>	AC005871	1,245 bp	23% FMLPR	P21462
5	<b>hRUP7</b>	AC007922	1,173 bp	48% GPR66	NP_006047
	<b>hCHN3</b>	EST 36581	1,113 bp	43% H3R	AF140538
	<b>hCHN4</b>	AA804531	1,077 bp	53% GPR27	
	<b>hCHN6</b>	EST 2134670	1,503 bp	32% thrombin	4503637
	<b>hCHN8</b>	EST 764455	1,029 bp	36% edg-1	NP_001391
				47% KIAA0001	D13626
10	<b>hCHN9</b>	EST 1541536	1,077 bp	41% LTB4R	NM_000752
	<b>hCHN10</b>	EST 1365839	1,055 bp	35% P2Y	NM_002563

Receptor homology is useful in terms of gaining an appreciation of a role of the receptors within the human body. As the patent document progresses, we will disclose techniques for mutating these receptors to establish non-endogenous, constitutively activated versions of these receptors.

The techniques disclosed herein have also been applied to other human, orphan GPCRs known to the art, as will be apparent as the patent document progresses.

### C. Receptor Screening

Screening candidate compounds against a non-endogenous, constitutively activated version of the human GPCRs disclosed herein allows for the direct identification of candidate compounds which act at this cell surface receptor, without requiring use of the receptor's endogenous ligand. By determining areas within the body where the endogenous version of human GPCRs disclosed herein is expressed and/or over-expressed, it is possible to determine related disease/disorder states which are associated with the expression and/or over-expression

of the receptor; such an approach is disclosed in this patent document.

With respect to creation of a mutation that may evidence constitutive activation of the human GPCR disclosed herein is based upon the distance from the proline residue at which is presumed to be located within TM6 of the GPCR; this algorithmic technique is disclosed  
5 in co-pending and commonly assigned patent document U.S. Serial Number 09/170,496, incorporated herein by reference. The algorithmic technique is not predicated upon traditional sequence "alignment" but rather a specified distance from the aforementioned TM6 proline residue. By mutating the amino acid residue located 16 amino acid residues from this residue (presumably located in the IC3 region of the receptor) to, most preferably, a lysine residue,  
10 such activation may be obtained. Other amino acid residues may be useful in the mutation at this position to achieve this objective.

**D. Disease/Disorder Identification and/or Selection**

As will be set forth in greater detail below, most preferably inverse agonists to the non-endogenous, constitutively activated GPCR can be identified by the methodologies of this  
15 invention. Such inverse agonists are ideal candidates as lead compounds in drug discovery programs for treating diseases related to this receptor. Because of the ability to directly identify inverse agonists to the GPCR, thereby allowing for the development of pharmaceutical compositions, a search for diseases and disorders associated with the GPCR is relevant. For example, scanning both diseased and normal tissue samples for the presence  
20 of the GPCR now becomes more than an academic exercise or one which might be pursued along the path of identifying an endogenous ligand to the specific GPCR. Tissue scans can be conducted across a broad range of healthy and diseased tissues. Such tissue scans provide a preferred first step in associating a specific receptor with a disease and/or disorder. *See, for*



*example*, co-pending application (docket number ARE-0050) for exemplary dot-blot and RT-PCR results of several of the GPCRs disclosed herein.

Preferably, the DNA sequence of the human GPCR is used to make a probe for (a) dot-blot analysis against tissue-mRNA, and/or (b) RT-PCR identification of the expression  
5 of the receptor in tissue samples. The presence of a receptor in a tissue source, or a diseased tissue, or the presence of the receptor at elevated concentrations in diseased tissue compared to a normal tissue, can be preferably utilized to identify a correlation with a treatment regimen, including but not limited to, a disease associated with that disease.

Receptors can equally well be localized to regions of organs by this technique. Based on  
10 the known functions of the specific tissues to which the receptor is localized, the putative functional role of the receptor can be deduced.

#### **E. Screening of Candidate Compounds**

##### **1. Generic GPCR screening assay techniques**

When a G protein receptor becomes constitutively active, it binds to a G protein (*e.g.*,  
15 Gq, Gs, Gi, Gz, Go) and stimulates the binding of GTP to the G protein. The G protein then acts as a GTPase and slowly hydrolyzes the GTP to GDP, whereby the receptor, under normal conditions, becomes deactivated. However, constitutively activated receptors continue to exchange GDP to GTP. A non-hydrolyzable analog of GTP, [<sup>35</sup>S]GTPγS, can be used to monitor enhanced binding to membranes which express constitutively activated receptors.  
20 It is reported that [<sup>35</sup>S]GTPγS can be used to monitor G protein coupling to membranes in the absence and presence of ligand. An example of this monitoring, among other examples well-known and available to those in the art, was reported by Traynor and Nahorski in 1995. The preferred use of this assay system is for initial screening of candidate compounds because the

system is generically applicable to all G protein-coupled receptors regardless of the particular G protein that interacts with the intracellular domain of the receptor.

## 2. Specific GPCR screening assay techniques

Once candidate compounds are identified using the "generic" G protein-coupled receptor assay (*i.e.*, an assay to select compounds that are agonists, partial agonists, or inverse agonists), further screening to confirm that the compounds have interacted at the receptor site is preferred. For example, a compound identified by the "generic" assay may not bind to the receptor, but may instead merely "uncouple" the G protein from the intracellular domain.

### a. *Gs, Gz and Gi.*

10 *Gs* stimulates the enzyme adenylyl cyclase. *Gi* (and *Gz* and *Go*), on the other hand, inhibit this enzyme. Adenylyl cyclase catalyzes the conversion of ATP to cAMP; thus, constitutively activated GPCRs that couple the *Gs* protein are associated with increased cellular levels of cAMP. On the other hand, constitutively activated GPCRs that couple *Gi* (or *Gz*, *Go*) protein are associated with decreased cellular levels of cAMP. *See, generally,*  
15 "Indirect Mechanisms of Synaptic Transmission," Chpt. 8, From Neuron To Brain (3<sup>rd</sup> Ed.) Nichols, J.G. et al eds. Sinauer Associates, Inc. (1992). Thus, assays that detect cAMP can be utilized to determine if a candidate compound is, *e.g.*, an inverse agonist to the receptor (*i.e.*, such a compound would decrease the levels of cAMP). A variety of approaches known  
20 in the art for measuring cAMP can be utilized; a most preferred approach relies upon the use of anti-cAMP antibodies in an ELISA-based format. Another type of assay that can be utilized is a whole cell second messenger reporter system assay. Promoters on genes drive the expression of the proteins that a particular gene encodes. Cyclic AMP drives gene expression by promoting the binding of a cAMP-responsive DNA binding protein or

transcription factor (CREB) that then binds to the promoter at specific sites called cAMP response elements and drives the expression of the gene. Reporter systems can be constructed which have a promoter containing multiple cAMP response elements before the reporter gene, e.g.,  $\beta$ -galactosidase or luciferase. Thus, a constitutively activated Gs-linked receptor causes  
5 the accumulation of cAMP that then activates the gene and expression of the reporter protein. The reporter protein such as  $\beta$ -galactosidase or luciferase can then be detected using standard biochemical assays (Chen et al. 1995).

*b. Go and Gq.*

10 Gq and Go are associated with activation of the enzyme phospholipase C, which in turn hydrolyzes the phospholipid  $PIP_2$ , releasing two intracellular messengers: diacylglycerol (DAG) and inistol 1,4,5-triphoisphate ( $IP_3$ ). Increased accumulation of  $IP_3$  is associated with activation of Gq- and Go-associated receptors. *See, generally, "Indirect Mechanisms of Synaptic Transmission," Chpt. 8, From Neuron To Brain (3<sup>rd</sup> Ed.) Nichols,*  
15 *J.G. et al eds. Sinauer Associates, Inc. (1992). Assays that detect  $IP_3$  accumulation can be utilized to determine if a candidate compound is, e.g., an inverse agonist to a Gq- or Go-associated receptor (i.e., such a compound would decrease the levels of  $IP_3$ ). Gq-associated receptors can also been examined using an AP1 reporter assay in that Gq-dependent phospholipase C causes activation of genes containing AP1 elements; thus, activated Gq-*  
20 *associated receptors will evidence an increase in the expression of such genes, whereby inverse agonists thereto will evidence a decrease in such expression, and agonists will evidence an increase in such expression. Commercially available assays for such detection are available.*

### 3. GPCR Fusion Protein

The use of an endogenous, constitutively activate orphan GPCR or a non-endogenous, constitutively activated orphan GPCR, for use in screening of candidate compounds for the direct identification of inverse agonists, agonists and partial agonists provide an interesting  
5 screening challenge in that, by definition, the receptor is active even in the absence of an endogenous ligand bound thereto. Thus, in order to differentiate between, *e.g.*, the non-endogenous receptor in the presence of a candidate compound and the non-endogenous receptor in the absence of that compound, with an aim of such a differentiation to allow for an understanding as to whether such compound may be an inverse agonist, agonist, partial  
10 agonist or have no affect on such a receptor, it is preferred that an approach be utilized that can enhance such differentiation. A preferred approach is the use of a GPCR Fusion Protein.

Generally, once it is determined that a non-endogenous orphan GPCR has been constitutively activated using the assay techniques set forth above (as well as others), it is possible to determine the predominant G protein that couples with the endogenous GPCR.  
15 Coupling of the G protein to the GPCR provides a signaling pathway that can be assessed. Because it is most preferred that screening take place by use of a mammalian expression system, such a system will be expected to have endogenous G protein therein. Thus, by definition, in such a system, the non-endogenous, constitutively activated orphan GPCR will continuously signal. In this regard, it is preferred that this signal be enhanced such that in the  
20 presence of, *e.g.*, an inverse agonist to the receptor, it is more likely that it will be able to more readily differentiate, particularly in the context of screening, between the receptor when it is contacted with the inverse agonist.

The GPCR Fusion Protein is intended to enhance the efficacy of G protein coupling

with the non-endogenous GPCR. The GPCR Fusion Protein is preferred for screening with a non-endogenous, constitutively activated GPCR because such an approach increases the signal that is most preferably utilized in such screening techniques. This is important in facilitating a significant "signal to noise" ratio; such a significant ratio is import preferred for  
5 the screening of candidate compounds as disclosed herein.

The construction of a construct useful for expression of a GPCR Fusion Protein is within the purview of those having ordinary skill in the art. Commercially available expression vectors and systems offer a variety of approaches that can fit the particular needs of an investigator. The criteria of importance for such a GPCR Fusion Protein construct is  
10 that the endogenous GPCR sequence and the G protein sequence both be in-frame (preferably, the sequence for the endogenous GPCR is upstream of the G protein sequence) and that the "stop" codon of the GPCR must be deleted or replaced such that upon expression of the GPCR, the G protein can also be expressed. The GPCR can be linked directly to the G protein, or there can be spacer residues between the two (preferably, no more than about 12,  
15 although this number can be readily ascertained by one of ordinary skill in the art). We have a preference (based upon convenience) of use of a spacer in that some restriction sites that are not used will, effectively, upon expression, become a spacer. Most preferably, the G protein that couples to the non-endogenous GPCR will have been identified prior to the creation of the GPCR Fusion Protein construct. Because there are only a few G proteins that have been  
20 identified, it is preferred that a construct comprising the sequence of the G protein (*i.e.*, a universal G protein construct) be available for insertion of an endogenous GPCR sequence therein; this provides for efficiency in the context of large-scale screening of a variety of different endogenous GPCRs having different sequences.

As noted above, constitutively activated GPCRs that couple to Gi, Gz and Go are expected to inhibit the formation of cAMP making assays based upon these types of GPCRs challenging (*i.e.*, the cAMP signal decreases upon activation thus making the direct identification of, *e.g.*, inverse agonists (which would further decrease this signal), interesting).

5 As will be disclosed herein, we have ascertained that for these types of receptors, it is possible to create a GPCR Fusion Protein that is not based upon the endogenous GPCR's endogenous G protein, in an effort to establish a viable cyclase-based assay. Thus, for example, a Gz coupled receptor such as H9, a GPCR Fusion Protein can be established that utilizes a Gs fusion protein - we believe that such a fusion construct, upon expression, "drives" or "forces"

10 the non-endogenous GPCR to couple with, *e.g.*, Gs rather than the "natural" Gz protein, such that a cyclase-based assay can be established. Thus, for Gi, Gz and Go coupled receptors, we prefer that that when a GPCR Fusion Protein is used and the assay is based upon detection of adenylyl cyclase activity, that the fusion construct be established with Gs (or an equivalent G protein that stimulates the formation of the enzyme adenylyl cyclase).

15 **F. Medicinal Chemistry**

Generally, but not always, direct identification of candidate compounds is preferably conducted in conjunction with compounds generated via combinatorial chemistry techniques, whereby thousands of compounds are randomly prepared for such analysis. Generally, the results of such screening will be compounds having unique core structures; thereafter, these

20 compounds are preferably subjected to additional chemical modification around a preferred core structure(s) to further enhance the medicinal properties thereof. Such techniques are known to those in the art and will not be addressed in detail in this patent document.

### G. Pharmaceutical compositions

Candidate compounds selected for further development can be formulated into pharmaceutical compositions using techniques well known to those in the art. Suitable pharmaceutically-acceptable carriers are available to those in the art; for example, see

5 Remington's Pharmaceutical Sciences, 16<sup>th</sup> Edition, 1980, Mack Publishing Co., (Oslo et al., eds.)

### H. Other Utility

Although a preferred use of the non-endogenous versions the human GPCRs disclosed herein may be for the direct identification of candidate compounds as inverse agonists,

10 agonists or partial agonists (preferably for use as pharmaceutical agents), these versions of human GPCRs can also be utilized in research settings. For example, *in vitro* and *in vivo* systems incorporating GPCRs can be utilized to further elucidate and understand the roles these receptors play in the human condition, both normal and diseased, as well as understanding the role of constitutive activation as it applies to understanding the signaling

15 cascade. The value in non-endogenous human GPCRs is that their utility as a research tool is enhanced in that, because of their unique features, non-endogenous human GPCRs can be used to understand the role of these receptors in the human body before the endogenous ligand therefor is identified. Other uses of the disclosed receptors will become apparent to those in the art based upon, *inter alia*, a review of this patent document.

20

### EXAMPLES

The following examples are presented for purposes of elucidation, and not limitation, of the present invention. While specific nucleic acid and amino acid sequences are disclosed herein, those of ordinary skill in the art are credited with the ability to make minor

modifications to these sequences while achieving the same or substantially similar results reported below. The traditional approach to application or understanding of sequence cassettes from one sequence to another (*e.g.* from rat receptor to human receptor or from human receptor A to human receptor B) is generally predicated upon sequence alignment techniques whereby the sequences are aligned in an effort to determine areas of commonality. The mutational approach disclosed herein does not rely upon this approach but is instead based upon an algorithmic approach and a positional distance from a conserved proline residue located within the TM6 region of human GPCRs. Once this approach is secured, those in the art are credited with the ability to make minor modifications thereto to achieve substantially the same results (*i.e.*, constitutive activation) disclosed herein. Such modified approaches are considered within the purview of this disclosure

**Example 1****ENDOGENOUS HUMAN GPCRS****1. Identification of Human GPCRs**

Certain of the disclosed endogenous human GPCRs were identified based upon a review of the GenBank™ database information. While searching the database, the following cDNA clones were identified as evidenced below (Table C).

**TABLE C**

Disclosed Human Orphan GPCRs	Accession Number	Complete DNA Sequence (Base Pairs)	Open Reading Frame (Base Pairs)	Nucleic Acid SEQ.ID. NO.	Amino Acid SEQ.ID. NO.
hARE-3	AL033379	111,389 bp	1,260 bp	1	2
hARE-4	AC006087	226,925 bp	1,119 bp	3	4
hARE-5	AC006255	127,605 bp	1,104 bp	5	6
hRUP3	AL035423	140,094 bp	1,005 bp	7	8



hRUP5	AC005849	169,144 bp	1,413 bp	9	10
hRUP6	AC005871	218,807 bp	1,245 bp	11	12
hRUP7	AC007922	158,858 bp	1,173 bp	13	14

Other disclosed endogenous human GPCRs were identified by conducting a BLAST™ search of EST database (dbest) using the following EST clones as query sequences. The following EST clones identified were then used as a probe to screen a human genomic library (Table D).

TABLE D

	Disclosed Human Orphan GPCRs	Query (Sequence)	EST Clone/ Accession No. Identified	Open Reading Frame (Base Pairs)	Nucleic Acid SEQ.ID.NO.	Amino Acid SEQ.ID.NO.
10	hGPCR27	Mouse GPCR27	AA775870	1,125 bp	17	18
	hARE-1	TDAG	1689643 AI090920	999 bp	19	20
15	hARE-2	GPCR27	68530 AA359504	1,122 bp	21	22
	hPPR1	Bovine PPR1	238667 H67224	1,053 bp	23	24
	hG2A	Mouse 1179426	<i>See Example 2(a), below</i>	1,113 bp	25	26
	hCHN3	N.A.	EST 36581 (full length)	1,113 bp	27	28
	hCHN4	TDAG	1184934 AA804531	1,077 bp	29	30
20	hCHN6	N.A.	EST 2134670 (full length)	1,503 bp	31	32
	hCHN8	KIAA0001	EST 764455	1,029 bp	33	34
	hCHN 9	1365839	EST 1541536	1,077 bp	35	36
	hCHN10	Mouse EST 1365839	Human 1365839	1,005 bp	37	38
	hRUP4	N.A.	AI307658	1,296 bp	39	40
25		N.A. = "not applicable".				

## 2. Full Length Cloning

### a. Human G2A

Mouse EST clone 1179426 was used to obtain a human genomic clone containing all

but three amino acid G2A coding sequences. The 5' of this coding sequence was obtained by using 5'RACE, and the template for PCR was Clontech's Human Spleen Marathon-Ready™ cDNA. The disclosed human G2A was amplified by PCR using the G2A cDNA specific primers for the first and second round PCR as shown in SEQ.ID.NO.: 41 and SEQ.ID.NO.:42

5 as follows:

5'-CTGTGTACAGCAGTTCGCAGAGTG-3' (SEQ.ID.NO.: 41; 1<sup>st</sup> round PCR)

5'-GAGTGCCAGGCAGAGCAGGTAGAC-3' (SEQ.ID.NO.: 42; second round PCR).

PCR was performed using Advantage GC Polymerase Kit (Clontech; manufacturing instructions will be followed), at 94°C for 30 sec followed by 5 cycles of 94°C for 5 sec and 72°C for 4 min; and 30 cycles of 94° for 5 sec and 70° for 4 min. An approximate 1.3 Kb PCR fragment was purified from agarose gel, digested with Hind III and Xba I and cloned into the expression vector pRC/CMV2 (Invitrogen). The cloned-insert was sequenced using the T7 Sequenase™ kit (USB Amersham; manufacturer instructions followed) and the sequence was compared with the presented sequence. Expression of the human G2A was detected by probing an RNA dot blot (Clontech; manufacturer instructions followed) with the P<sup>32</sup>-labeled fragment.

**b. CHN9**

Sequencing of the EST clone 1541536 showed CHN9 to be a partial cDNA clone having only an initiation codon; *i.e.*, the termination codon was missing. When CHN9 was used to blast against data base (nr), the 3' sequence of CHN9 was 100% homologous to the 5' untranslated region of the leukotriene B4 receptor cDNA, which contained a termination codon in the frame with CHN9 coding sequence. To determine whether the 5' untranslated region of LTB4R cDNA was the 3' sequence of CHN9, PCR was performed using primers based upon the 5' sequence flanking the initiation codon found in CHN9 and

the 3' sequence around the termination codon found in the LTB4R 5' untranslated region.

The 5' primer sequence utilized was as follows:

5'-CCCGAATTCCTGCTTGCTCCCAGCTTGGCCC-3' (SEQ.ID.NO.: 43; sense) and

5'-TGTGGATCCTGCTGTCAAAGGTCCCATTCGG-3' (SEQ.ID.NO.: 44; antisense).

- 5 PCR was performed using thymus cDNA as a template and rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer, 0.25 uM of each primer, and 0.2 mM of each 4 nucleotides. The cycle condition was 30 cycles of 94°C for 1 min, 65°C for 1min and 72 °C for 1 min and 10 sec. A 1.1kb fragment consistent with the predicted size was obtained from PCR. This PCR fragment was subcloned into pCMV (*see below*) and
- 10 sequenced (*see*, SEQ.ID.NO.: 35).

#### c. RUP 4

The full length RUP4 was cloned by RT-PCR with human brain cDNA (Clontech) as templates:

5'-TCACAATGCTAGGTGTGGTC-3' (SEQ.ID.NO.: 45; sense) and

- 15 5'-TGCATAGACAATGGGATTACAG-3' (SEQ.ID.NO.: 46; antisense).

PCR was performed using TaqPlus Precision™ polymerase (Stratagene; manufacturing instructions followed) by the following cycles: 94°C for 2 min; 94°C 30 sec; 55°C for 30 sec, 72°C for 45 sec, and 72°C for 10 min. Cycles 2 through 4 were repeated 30 times.

- The PCR products were separated on a 1% agarose gel and a 500 bp PCR fragment
- 20 was isolated and cloned into the pCRII-TOPO™ vector (Invitrogen) and sequenced using the T7 DNA Sequenase™ kit (Amsham) and the SP6/T7 primers (Stratagene). Sequence analysis revealed that the PCR fragment was indeed an alternatively spliced form of AI307658 having a continuous open reading frame with similarity to other GPCRs. The completed sequence of this PCR fragment was as follows:

5'-TCACAATGCTAGGTGTGGTCTGGCTGGTGGCAGTCATCGTAGGATCACCCATGTGGCAC  
GTGCAACAACTTGAGATCAAATATGACTTCCTATATGAAAAGGAACACATCTGCTGCTTAAGA  
GTGGACCAGCCCTGTGCACCAGAAGATCTACACCACCTTCATCCTTGTATCCTCTTCCTCCTGC  
CTCTTATGGTGATGCTTATTCTGTACGTAATAATTGGTTATGAACTTTGGATAAAGAAAAGAGTT  
5 GGGGATGGTTCAGTGCTTCGAACTATTCATGGAAAAGAAATGTCCAAAATAGCCAGGAAGAAG  
AAACGAGCTGTCAATTATGATGGTGACAGTGGTGGCTCTCTTTGCTGTGTGCTGGGCACCATTC  
ATGTTGTCCATATGATGATTGAATACAGTAATTTTGAAAAGGAATATGATGATGTCACAATCAA  
GATGATTTTGTCTATCGTGCAAATTATTGGATTTTCCAACCTCCATCTGTAATCCCATTTGTCTATGCA-  
3' (SEQ.ID.NO.: 47)

10 Based on the above sequence, two sense oligonucleotide primer sets:

5'-CTGCTTAGAAGAGTGGACCAG-3' (SEQ.ID.NO.: 48; oligo 1),

5'-CTGTGCACCAGAAGATCTACAC-3' (SEQ.ID.NO.: 49; oligo 2) and

two antisense oligonucleotide primer sets:

5'-CAAGGATGAAGGTGGTGTAGA-3' (SEQ.ID.NO.: 50; oligo 3)

15 5'-GTGTAGATCTTCTGGTGCACAGG-3' (SEQ.ID.NO.: 51; oligo 4)

were used for 3'- and 5'-RACE PCR with a human brain Marathon-Ready™ cDNA (Clontech, Cat# 7400-1) as template, according to manufacture's instructions. DNA fragments generated by the RACE PCR were cloned into the pCRII-TOPO™ vector (Invitrogen) and sequenced using the SP6/T7 primers (Stratagene) and some internal primers.

20 The 3' RACE product contained a poly(A) tail and a completed open reading frame ending at a TAA stop codon. The 5' RACE product contained an incomplete 5' end; *i.e.*, the ATG initiation codon was not present.

Based on the new 5' sequence, oligo 3 and the following primer:

5'-GCAATGCAGGTCATAGTGAGC -3' (SEQ.ID.NO.: 52; oligo 5)

25 were used for the second round of 5' race PCR and the PCR products were analyzed as above.

A third round of 5' race PCR was carried out utilizing antisense primers:

5'-TGGAGCATGGTGACGGGAATGCAGAAG-3' (SEQ.ID.NO.: 53; oligo 6) and

5'-GTGATGAGCAGGTCAGTACGCGCAAG-3' (SEQ.ID.NO.: 54; oligo 7).

The sequence of the 5' RACE PCR products revealed the presence of the initiation codon

ATG, and further round of 5' race PCR did not generate any more 5' sequence. The completed 5' sequence was confirmed by RT-PCR using sense primer 5'-GCAATGCAGGCGCTTAACATTAC-3' (SEQ.ID.NO.: 55; oligo 8) and oligo 4 as primers and sequence analysis of the 650 bp PCR product generated from human brain and heart cDNA templates (Clontech, Cat# 7404-1). The completed 3' sequence was confirmed by RT-PCR using oligo 2 and the following antisense primer:

5'-TTGGGTTACAATCTGAAGGGCA-3' (SEQ.ID.NO.:56; oligo 9)

and sequence analysis of the 670 bp PCR product generated from human brain and heart cDNA templates. (Clontech, Cat# 7404-1).

10

#### d. RUP5

The full length RUP5 was cloned by RT-PCR using a sense primer upstream from ATG, the initiation codon (SEQ.ID.NO.:57), and an antisense primer containing TCA as the stop codon (SEQ.ID.NO.:58), which had the following sequences:

5'-ACTCCGTGTCCAGCAGGACTCTG-3' (SEQ.ID.NO.: 57)

15 5'-TGCGTGTTCTGACCCCTCACGTG-3' (SEQ.ID.NO.: 58)

and human peripheral leukocyte cDNA (Clontech) as a template. Advantage™ cDNA polymerase (Clontech) was used for the amplification in a 50ul reaction by the following cycle with step 2 through step 4 repeated 30 times: 94°C for 30 sec; 94° for 15 sec; 69° for 40 sec; 72°C for 3 min; and 72°C fro 6 min. A 1.4kb PCR fragment was isolated and cloned with the pCRII-TOPO™ vector (Invitrogen) and completely sequenced using the T7 DNA Sequenase™ kit (Amsham). See, SEQ.ID.NO.: 9.

#### e. RUP6

The full length RUP6 was cloned by RT-PCR using primers:

5'-CAGGCCTTGGATTTTAATGTCAGGGATGG-3' (SEQ.ID.NO.: 59) and

5'-GGAGAGTCAGCTCTGAAAGAATTCAGG-3' (SEQ.ID.NO.: 60);

and human thymus Marathon-Ready™ cDNA (Clontech) as a template. Advantage cDNA polymerase (Clontech, according to manufacturer's instructions) was used for the amplification in a 50ul reaction by the following cycle: 94°C for 30sec; 94°C for 5 sec; 66°C  
5 for 40sec; 72°C for 2.5 sec and 72°C for 7 min. Cycles 2 through 4 were repeated 30 times. A 1.3 Kb PCR fragment was isolated and cloned into the pCRII-TOPO™ vector (Invitrogen) and completely sequenced (*see*, SEQ.ID.NO.: 11) using the ABI Big Dye Terminator™ kit (P.E. Biosystem).

#### f. RUP7

10 The full length RUP7 was cloned by RT-PCR using primers:

5'-TGATGTGATGCCAGATACTAATAGCAC-3' (SEQ.ID.NO.: 61; sense) and

5'-CCTGATTCATTTAGGTGAGATTGAGAC-3' (SEQ.ID.NO.: 62; antisense)

and human peripheral leukocyte cDNA (Clontech) as a template. Advantage™ cDNA polymerase (Clontech) was used for the amplification in a 50 ul reaction by the following  
15 cycle with step 2 to step 4 repeated 30 times: 94°C for 2 minutes; 94°C for 15 seconds; 60°C for 20 seconds; 72°C for 2 minutes; 72°C for 10 minutes. A 1.25 Kb PCR fragment was isolated and cloned into the pCRII-TOPO™ vector (Invitrogen) and completely sequenced using the ABI Big Dye Terminator™ kit (P.E. Biosystem). *See*, SEQ.ID.NO.: 13.

#### 3. Angiotensin II Type 1 Receptor ("AT1")

20 The endogenous human angiotensin II type 1 receptor ("AT1") was obtained by PCR using genomic DNA as template and rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer, 0.25 µM of each primer, and 0.2 mM of each 4 nucleotides. The cycle condition was 30 cycles of 94°C for 1 min, 55°C for 1min and 72 °C for 1.5 min. The 5' PCR primer contains a HindIII site with the sequence:

5'-CCCAAGCTTCCCCAGGTGTATTTGAT-3' (SEQ.ID.NO.: 63)

and the 3' primer contains a BamHI site with the following sequence:

5'-GTTGGATCCACATAATGCATTTTCTC-3' (SEQ.ID.NO.: 64).

The resulting 1.3 kb PCR fragment was digested with HindIII and BamHI and cloned into  
5 HindIII-BamHI site of pCMV expression vector. The cDNA clone was fully sequenced.  
Nucleic acid (SEQ.ID.NO.: 65) and amino acid (SEQ.ID.NO.: 66) sequences for human AT1  
were thereafter determined and verified.

#### 4. GPR38

To obtain GPR38, PCR was performed by combining two PCR fragments, using  
10 human genomic cDNA as template and rTth polymerase (Perkin Elmer) with the buffer system  
provided by the manufacturer, 0.25uM of each primer, and 0.2 mM of each 4 nucleotides.  
The cycle condition for each PCR reaction was 30 cycles of 94°C for 1 min, 62°C for 1min  
and 72°C for 2 min.

The first fragment was amplified with the 5' PCR primer that contained an end site  
15 with the following sequence:

5'-ACCATGGGCAGCCCCTGGAACGGCAGC-3' (SEQ.ID.NO.:67)

and a 3' primer having the following sequence:

5'-AGAACCACCACCAGCAGGACGCGGACGGTCTGCCGGTGG-3' (SEQ.ID.NO.:68).

The second PCR fragment was amplified with a 5' primer having the following sequence:

20 5'-GTCCGCGTCCTGCTGGTGGTGGTTCTGGCATTATAATT-3' (SEQ.ID.NO.: 69)

and a 3' primer that contained a BamHI site and having the following sequence:

5'-CCTGGATCCTTATCCCATCGTCTCACGTTAGC-3' (SEQ.ID.NO.: 70).

The two fragments were used as templates to amplify GPR38, using SEQ.ID.NO.: 67 and  
SEQ.ID.NO.: 70 as primers (using the above-noted cycle conditions). The resulting 1.44kb

PCR fragment was digested with BamHI and cloned into Blunt-BamHI site of pCMV expression vector.

#### 5. MC4

To obtain MC4, PCR was performed using human genomic cDNA as template and  
5 rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer, 0.25uM of each primer, and 0.2 mM of each 4 nucleotides. The cycle condition for each PCR reaction was 30 cycles of 94°C for 1 min, 54°C for 1min and 72°C for 1.5 min.

The 5' PCR contained an EcoRI site with the sequence:

5'-CTGGAATTCTCCTGCCAGCATGGTGA-3' (SEQ.ID.NO.: 71)

10 and the 3' primer contained a BamHI site with the sequence:

5'-GCAGGATCCTATATTGCGTGCTCTGTCCCC'-3 (SEQ.ID.NO.: 72).

The 1.0 kb PCR fragment was digest with EcoRI and BamHI and cloned into EcoRI-BamHI site of pCMV expression vector. Nucleic acid (SEQ.ID.NO.: 73) and amino acid (SEQ.ID.NO.: 74) sequences for human MC4 were thereafter determined.

15

#### 6. CCKB

To obtain CCKB, PCR was performed using human stomach cDNA as template and rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer, 0.25uM of each primer, and 0.2 mM of each 4 nucleotides. The cycle condition for each PCR reaction was 30 cycles of 94°C for 1 min, 65°C for 1min and 72°C for 1 min and 30 sec.

20 The 5' PCR contained a HindIII site with the sequence:

5'-CCGAAGCTTCGAGCTGAGTAAGGCGGCGGGCT-3' (SEQ.ID.NO.: 75)

and the 3' primer contained an EcoRI site with the sequence:

5'-GTGGAATTCATTTGCCCTGCCTCAACCCCCA-3 (SEQ.ID.NO.: 76).

The resulting 1.44 kb PCR fragment was digest with HindIII and EcoRI and cloned into



HindIII-EcoRI site of pCMV expression vector. Nucleic acid (SEQ.ID.NO.: 77) and amino acid (SEQ.ID.NO.: 78) sequences for human CCKB were thereafter determined.

## 7. TDAG8

To obtain TDAG8, PCR was performed using genomic DNA as template and rTth  
5 polymerase (Perkin Elmer) with the buffer system provided by the manufacturer, 0.25  $\mu$ M of each primer, and 0.2 mM of each 4 nucleotides. The cycle condition was 30 cycles of 94°C for 1 min, 56°C for 1min and 72 °C for 1 min and 20 sec. The 5' PCR primer contained a HindIII site with the following sequence:

5'-TGCAAGCTTAAAAAGGAAAAAATGAACAGC-3' (SEQ.ID.NO.: 79)

10 and the 3' primer contained a BamHI site with the following sequence:

5'-TAAGGATCCCTTCCCTTCAAAACATCCTTG -3' (SEQ.ID.NO.: 80).

The resulting 1.1 kb PCR fragment was digested with HindIII and BamHI and cloned into HindIII-BamHI site of pCMV expression vector. Three resulting clones sequenced contained three potential polymorphisms involving changes of amino acid 43 from Pro to Ala, amino  
15 acid 97 from Lys to Asn and amino acid 130 from Ile to Phe. Nucleic acid (SEQ.ID.NO.: 81) and amino acid (SEQ.ID.NO.: 82) sequences for human TDAG8 were thereafter determined.

## 8. H9

To obtain H9, PCR was performed using pituitary cDNA as template and rTth  
20 polymerase (Perkin Elmer) with the buffer system provided by the manufacturer, 0.25  $\mu$ M of each primer, and 0.2 mM of each 4 nucleotides. The cycle condition was 30 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 2 min. The 5' PCR primer contained a HindIII site with the following sequence:

5'-GGAAAGCTTAACGATCCCCAGGAGCAACAT-3' (SEQ.ID.NO.:15)

and the 3' primer contained a BamHI site with the following sequence:

5'-CTGGGATCCTACGAGAGCATTTTTCACACAG-3' (SEQ.ID.NO.:16).

The resulting 1.9 kb PCR fragment was digested with HindIII and BamHI and cloned into HindIII-BamHI site of pCMV expression vector. H9 contained three potential polymorphisms involving changes of amino acid P320S, S493N and amino acid G448A. Nucleic acid  
5 (SEQ.ID.NO.: 139) and amino acid (SEQ.ID.NO.: 140) sequences for human H9 were thereafter determined and verified.

### Example 2

#### PREPARATION OF NON-ENDOGENOUS, CONSTITUTIVELY ACTIVATED GPCRS

Those skilled in the art are credited with the ability to select techniques for  
10 mutation of a nucleic acid sequence. Presented below are approaches utilized to create non-endogenous versions of several of the human GPCRs disclosed above. The mutations disclosed below are based upon an algorithmic approach whereby the 16<sup>th</sup> amino acid (located in the IC3 region of the GPCR) from a conserved proline residue (located in the TM6 region of the GPCR, near the TM6/IC3 interface) is mutated, most preferably to a  
15 lysine amino acid residue.

#### 1. Transformer Site-Directed™ Mutagenesis

Preparation of non-endogenous human GPCRs may be accomplished on human GPCRs using Transformer Site-Directed™ Mutagenesis Kit (Clontech) according to the manufacturer instructions. Two mutagenesis primers are utilized, most preferably a lysine  
20 mutagenesis oligonucleotide that creates the lysine mutation, and a selection marker oligonucleotide. For convenience, the codon mutation to be incorporated into the human GPCR is also noted, in standard form (Table E):

TABLE E

	Receptor Identifier	Codon Mutation
	hARE-3	F313K
	hARE-4	V233K
5	hARE-5	A240K
	hGPCR14	L257K
	hGPCR27	C283K
	hARE-1	E232K
	hARE-2	G285K
10	hPPR1	L239K
	hG2A	K232A
	hRUP3	L224K
	hRUP5	A236K
	hRUP6	N267K
15	hRUP7	A302K
	hCHN4	V236K
	hMC4	A244K
	hCHN3	S284K
	hCHN6	L352K
20	hCHN8	N235K
	hCHN9	G223K
	hCHN10	L231K
	hH9	F236K

The following GPCRs were mutated according with the above method using the  
designated sequence primers (Table F).

**TABLE F**

Receptor Identifier	Codon Mutation	Lysine Mutagenesis (SEQ.ID.NO.) 5'-3' orientation, mutation sequence underlined	Selection Marker (SEQ.ID.NO.) 5'-3' orientation
hRUP4	V272K	CAGGAAGAAGAAACGAGC TGTCATTATGATGGTGACA GTG (83)	CACTGTCACCATCATAATG ACAGCTCGTTTCTTCTTCC TG (84)
5 hAT1	see below	alternative approach; see below	alternative approach; see below
hGPR38	V297K	GGCCACCGGCAGACCAAAAC GCGTCCTGCTG (85)	CTCCTTCGGTCCTCCTATC GTTGTCAGAAAGT (86)
hCCKB	V332K	alternative approach; see below	alternative approach; see below
hTDAG8	I225K	GGAAAAGAAGAGAATCAA AAAACTACTTGTCAGCATC (87)	CTCCTTCGGTCCTCCTATC GTTGTCAGAAAGT (88)
hH9	F236K	GCTGAGGTTTCGCAATAAAAC TAACCATGTTTGTG (143)	CTCCTTCGGTCCTCCTATC GTTGTCAGAAAGT (144)
hMC4	A244K	GCCAATATGAAGGGAAAA ATTACCTTGACCATC (137)	CTCCTTCGGTCCTCCTATC GTTGTCAGAAAGT (138)

- 10 The non-endogenous human GPCRs were then sequenced and the derived and verified nucleic acid and amino acid sequences are listed in the accompanying "Sequence Listing" appendix to this patent document, as summarized in Table G below:

**TABLE G**

	Non Endogenous Human GPCR	Nucleic Acid Sequence Listing	Amino Acid Sequence Listing
15	hRUP4 (V272K)	SEQ.ID.NO.: 127	SEQ.ID.NO.: 128
	hAT1 (see alternative approaches below)	(see alternative approaches below)	(see alternative approaches, below)
20	hGPR38 (V297K)	SEQ.ID.NO.: 129	SEQ.ID.NO.: 130
	hCCKB (V332K)	SEQ.ID.NO.: 131	SEQ.ID.NO.: 132
25	hTDAG8 (I225K)	SEQ.ID.NO.: 133	SEQ.ID.NO.: 134
	hH9 (F236K)	SEQ.ID.NO.: 141	SEQ.ID.NO.: 142
30	hMC4 (A244K)	SEQ.ID.NO.: 135	SEQ.ID.NO.: 136

## 2. Alternative Approaches For Creation of Non-Endogenous Human GPCRs

### a. AT1

5

#### 1. F239K Mutation

Preparation of a non-endogenous, constitutively activated human AT1 receptor was accomplished by creating an F239K mutation (see, SEQ.ID.NO.: 89 for nucleic acid sequence, and SEQ.ID.NO.: 90 for amino acid sequence). Mutagenesis was performed using Transformer Site-Directed Mutagenesis™ Kit (Clontech) according to the to manufacturer's instructions. The two mutagenesis primers were used, a lysine mutagenesis oligonucleotide (SEQ.ID.NO.: 91) and a selection marker oligonucleotide (SEQ.ID.NO.: 92), which had the following sequences:

5'-CCAAGAAATGATGATATTAAGATAATTATGGC-3' (SEQ.ID.NO.: 91)

5'-CTCCTTCGGTCCTCTATCGTTGTCAGAAGT-3' (SEQ.ID.NO.: 92),

15 respectively.

#### 2. N111A Mutation

Preparation of a non-endogenous human AT1 receptor was also accomplished by creating an N111A mutation (see, SEQ.ID.NO.:93 for nucleic acid sequence, and SEQ.ID.NO.: 94 for amino acid sequence). Two PCR reactions were performed using pfu polymerase (Stratagene) with the buffer system provided by the manufacturer, supplemented with 10% DMSO, 0.25 μM of each primer, and 0.5 mM of each 4 nucleotides. The 5' PCR sense primer used had the following sequence:

5'-CCCAAGCTTCCCCAGGTGTATTTGAT-3' (SEQ.ID.NO.: 95)

25 and the antisense primer had the following sequence:

5'-CCTGCAGGCGAAACTGACTCTGGCTGAAG-3' (SEQ.ID.NO.: 96).

The resulting 400 bp PCR fragment was digested with HindIII site and subcloned into HindIII-SmaI site of pCMV vector (5' construct). The 3' PCR sense primer used had the following sequence:

5 5'-CTGTACGCTAGTGTGTTTCTACTCACGTGTCTCAGCATTGAT-3' (SEQ.ID.NO.: 97)

and the antisense primer had the following sequence:

5'-GTTGGATCCACATAATGCATTTTCTC-3' (SEQ.ID.NO.: 98)

The resulting 880 bp PCR fragment was digested with BamHI and inserted into Pst (blunted by T4 polymerase) and BamHI site of 5' construct to generated the full length  
10 N111A construct. The cycle condition was 25 cycles of 94°C for 1 min, 60°C for 1min and 72 °C for 1 min (5' PCR) or 1.5 min (3' PCR).

### 3. AT2K255IC3 Mutation

Preparation of a non-endogenous, constitutively activated human AT1 was accomplished by creating an AT2K255IC3 "domain swap" mutation (see, SEQ.ID.NO.:99  
15 for nucleic acid sequence, and SEQ.ID.NO.: 100 for amino acid sequence). Restriction sites flanking IC3 of AT1 were generated to facilitate replacement of the IC3 with corresponding IC3 from angiotensin II type 2 receptor (AT2). This was accomplished by performing two PCR reactions. A 5' PCR fragment (Fragment A) encoded from the 5' untranslated region to the beginning of IC3 was generated by utilizing SEQ.ID.NO.: 63 as  
20 sense primer and the following sequence:

5'-TCCGAATTCCAAAATAACTTGTAAGAATGATCAGAAA-3' (SEQ.ID.NO.: 101)

as antisense primer. A 3' PCR fragment (Fragment B) encoding from the end of IC3 to the 3' untranslated region was generated by using the following sequence:

5'-AGATCTTAAGAAGATAATTATGGCAATTGTGCT-3' (SEQ.ID.NO.: 102)

as sense primer and SEQ.ID.NO.: 64 as antisense primer. The PCR condition was 30 cycles of 94°C for 1 min, 55°C for 1min and 72 °C for 1.5 min using endogenous AT1 cDNA clone as template and pfu polymerase (Stratagene), with the buffer systems provided by the manufacturer, supplemented with 10% DMSO, 0.25 µM of each primer, and 0.5 mM of each 4 nucleotides. Fragment A (720 bp) was digested with HindIII and EcoRI and subcloned. Fragment B was digested with BamHI and subcloned into pCMV vector with an EcoRI site 5' to the cloned PCR fragment.

The DNA fragment (Fragment C) encoding IC3 of AT2 with a L255K mutation and containing an EcoRI cohesive end at 5' and a AflII cohesive end at 3', was generated by annealing 2 synthetic oligonucleotides having the following sequences:

5'AATTCGAAAACACTTACTGAAGACGAATAGCTATGGGAAGAACAGGATAACCCGTGACCAAG-3' (sense; SEQ.ID.NO.: 103)

5'TTAAC TTGGTCACGGGTTATCCTGTTCTTCCCATAGCTATTCGTCTTCAGT AAGTGT TTTTCG-3' (antisense; SEQ.ID.NO.: 104).

Fragment C was inserted in front of Fragment B through EcoRI and AflII site. The resulting clone was then ligated with the Fragment A through the EcoRI site to generate AT1 with AT2K255IC3.

#### 4. A243+ Mutation

Preparation of a non-endogenous human AT1 receptor was also accomplished by creating an A243+ mutation (see, SEQ.ID.NO.: 105 for nucleic acid sequence, and SEQ.ID.NO.: 106 for amino acid sequence). An A243+ mutation was constructed using the following PCR based strategy: Two PCR reactions was performed using pfu polymerase (Stratagene) with the buffer system provided by the manufacturer supplemented with 10% DMSO, 0.25 µM of each primer, and 0.5 mM of each 4 nucleotides. The 5' PCR sense primer

5'-CCCAAGCTTCCCCAGGTGTATTTGAT-3' (SEQ.ID.NO.: 107)

and the antisense primer had the following sequence:

5'-AAGCACAATTGCTGCATAATTATCTTAAAAATATCATC-3' (SEQ.ID.NO.: 108).

5 The 3' PCR sense primer utilized had the following sequence:

5'-AAGATAATTATGGCAGCAATTGTGCTTTTCTTTTCTTT-3' (SEQ.ID.NO.: 109)

containing the Ala insertion and antisense primer:

5'-GTTGGATCCACATAATGCATTTTCTC-3'(SEQ.ID.NO.: 110).

The cycle condition was 25 cycles of 94°C for 1 min, 54°C for 1min and 72 °C for 1.5 min.

10 An aliquot of the 5' and 3' PCR were then used as co-template to perform secondary PCR using the 5' PCR sense primer and 3' PCR antisense primer. The PCR condition was the same as primary PCR except the extension time was 2.5 min. The resulting PCR fragment was digested with HindIII and BamHI and subcloned into pCMV vector. (See, SEQ.ID.NO.: 105)

15 **4. CCKB**

Preparation of the non-endogenous, constitutively activated human CCKB receptor was accomplished by creating a V322K mutation (see, SEQ.ID.NO.: 111 for nucleic acid sequence and SEQ.ID.NO.: 112 for amino acid sequence). Mutagenesis was performed by PCR via amplification using the wildtype CCKB from Example 1.

20 The first PCR fragment (1kb) was amplified by using SEQ.ID.NO.: 75 and an antisense primer comprising a V322K mutation:

5'-CAGCAGCATGCGCTTCACGCGCTTCTTAGCCCAG-3' (SEQ.ID.NO.: 113).

The second PCR fragment (0.44kb) was amplified by using a sense primer comprising the V322K mutation:



5'-AGAAGCGCGTGAAGCGCATGCTGCTGGTGATCGTT-3' (SEQ.ID.NO.: 114) and SEQ.ID.NO.: 76.

The two resulting PCR fragments were then used as template for amplifying CCKB comprising V332K, using SEQ.ID.NO.: 75 and SEQ.ID.NO.: 76 and the above-noted  
5 system and conditions. The resulting 1.44kb PCR fragment containing the V332K mutation was digested with HindIII and EcoRI and cloned into HindIII-EcoRI site of pCMV expression vector. (See, SEQ.ID.NO.: 111).

### 3. QuikChange™ Site-Directed™ Mutagenesis

Preparation of non-endogenous human GPCRs can also be accomplished by using  
10 QuikChange™ Site-Directed™ Mutagenesis Kit (Stratagene, according to manufacturer's instructions). Endogenous GPCR is preferably used as a template and two mutagenesis primers utilized, as well as, most preferably, a lysine mutagenesis oligonucleotide and a selection marker oligonucleotide (included in kit). For convenience, the codon mutation incorporated into the human GPCR and the respective oligonucleotides are noted, in standard  
15 form (Table H):

**TABLE H**

Receptor Identifier	Codon Mutation	Lysine Mutagenesis (SEQ.ID.NO.) 5'-3' orientation, mutation underlined	Selection Marker (SEQ.ID.NO.) 5'-3' orientation
hCHN3	S284K	ATGGAGAAAAGAATCA <u>AAA</u> AGAA TGTTCTATATA (115)	TATATAGAACATTCTTTT GATTCTTTTCTCCAT (116)
hCHN6	L352K	CGCTCTCTGGCCTTGAAGCGCAC GCTCAGC (117)	GCTGAGCGTGCGCTTCA AGGCCAGAGAGCG (118)
5 hCHN8	N235K	CCCAGGAAAAAGGTGA <u>AA</u> GTCA AAGTTTTC (119)	GAAAACTTTGACTTTTCAC CTTTTTCCTGGG (120)
hCHN9	G223K	GGGGCGCGGGTGAAACGGCTGG TGAGC (121)	GCTCACCAGCCGTTTCA CCGCGCCCC (122)
hCHN10	L231K	CCCCTTGAAAGCCTAAGAACTT GGTCATC (123)	GATGACCAAGTTCTTAG GCTTTTCAAGGGG (124)

### Example 3 RECEPTOR EXPRESSION

10           Although a variety of cells are available to the art for the expression of proteins, it is most preferred that mammalian cells be utilized. The primary reason for this is predicated upon practicalities, *i.e.*, utilization of, *e.g.*, yeast cells for the expression of a GPCR, while possible, introduces into the protocol a non-mammalian cell which may not (indeed, in the case of yeast, does not) include the receptor-coupling, genetic-mechanism and secretary

15 pathways that have evolved for mammalian systems - thus, results obtained in non-mammalian cells, while of potential use, are not as preferred as that obtained from mammalian cells. Of the mammalian cells, COS-7, 293 and 293T cells are particularly preferred, although the specific mammalian cell utilized can be predicated upon the particular needs of the artisan.

On day one,  $1 \times 10^7$  293T cells per 150mm plate were plated out. On day two, two

20 reaction tubes were prepared (the proportions to follow for each tube are per plate): tube A was prepared by mixing 20 $\mu$ g DNA (*e.g.*, pCMV vector; pCMV vector with receptor cDNA, etc.) in 1.2ml serum free DMEM (Irvine Scientific, Irvine, CA); tube B was

prepared by mixing 120 $\mu$ l lipofectamine (Gibco BRL) in 1.2ml serum free DMEM. Tubes A and B were admixed by inversions (several times), followed by incubation at room temperature for 30-45min. The admixture is referred to as the "transfection mixture".

Plated 293T cells were washed with 1XPBS, followed by addition of 10ml serum free  
5 DMEM. 2.4ml of the transfection mixture were added to the cells, followed by incubation for 4hrs at 37°C/5% CO<sub>2</sub>. The transfection mixture was removed by aspiration, followed by the addition of 25ml of DMEM/10% Fetal Bovine Serum. Cells were incubated at 37°C/5% CO<sub>2</sub>. After 72hr incubation, cells were harvested and utilized for analysis.

#### Example 4

#### 10 ASSAYS FOR DETERMINATION OF CONSTITUTIVE ACTIVITY OF NON-ENDOGENOUS GPCRS

A variety of approaches are available for assessment of constitutive activity of the non-endogenous human GPCRs. The following are illustrative; those of ordinary skill in the art are credited with the ability to determine those techniques that are preferentially  
15 beneficial for the needs of the artisan.

##### 1. Membrane Binding Assays: [<sup>35</sup>S]GTP $\gamma$ S Assay

When a G protein-coupled receptor is in its active state, either as a result of ligand binding or constitutive activation, the receptor couples to a G protein and stimulates the release of GDP and subsequent binding of GTP to the G protein. The alpha subunit of the G  
20 protein-receptor complex acts as a GTPase and slowly hydrolyzes the GTP to GDP, at which point the receptor normally is deactivated. Constitutively activated receptors continue to exchange GDP for GTP. The non-hydrolyzable GTP analog, [<sup>35</sup>S]GTP $\gamma$ S, can be utilized to demonstrate enhanced binding of [<sup>35</sup>S]GTP $\gamma$ S to membranes expressing constitutively activated receptors. The advantage of using [<sup>35</sup>S]GTP $\gamma$ S binding to measure constitutive

activation is that: (a) it is generically applicable to all G protein-coupled receptors; (b) it is proximal at the membrane surface making it less likely to pick-up molecules which affect the intracellular cascade.

The assay utilizes the ability of G protein coupled receptors to stimulate [<sup>35</sup>S]GTPγS  
5 binding to membranes expressing the relevant receptors. The assay can, therefore, be used in the direct identification method to screen candidate compounds to known, orphan and constitutively activated G protein-coupled receptors. The assay is generic and has application to drug discovery at all G protein-coupled receptors.

The [<sup>35</sup>S]GTPγS assay can be incubated in 20 mM HEPES and between 1 and about  
10 20mM MgCl<sub>2</sub> (this amount can be adjusted for optimization of results, although 20mM is preferred) pH 7.4, binding buffer with between about 0.3 and about 1.2 nM [<sup>35</sup>S]GTPγS (this amount can be adjusted for optimization of results, although 1.2 is preferred ) and 12.5 to 75 μg membrane protein (*e.g.* COS-7 cells expressing the receptor; this amount can be adjusted for optimization, although 75μg is preferred) and 1 μM GDP (this amount can be changed for  
15 optimization) for 1 hour. Wheatgerm agglutinin beads (25 μl; Amersham) should then be added and the mixture incubated for another 30 minutes at room temperature. The tubes are then centrifuged at 1500 x g for 5 minutes at room temperature and then counted in a scintillation counter.

A less costly but equally applicable alternative has been identified which also meets  
20 the needs of large scale screening. Flash plates™ and Wallac™ scintistrips may be utilized to format a high throughput [<sup>35</sup>S]GTPγS binding assay. Furthermore, using this technique, the assay can be utilized for known GPCRs to simultaneously monitor tritiated ligand binding to the receptor at the same time as monitoring the efficacy via [<sup>35</sup>S]GTPγS binding. This is

possible because the Wallac beta counter can switch energy windows to look at both tritium and  $^{35}\text{S}$ -labeled probes. This assay may also be used to detect other types of membrane activation events resulting in receptor activation. For example, the assay may be used to monitor  $^{32}\text{P}$  phosphorylation of a variety of receptors (both G protein coupled and tyrosine kinase receptors). When the membranes are centrifuged to the bottom of the well, the bound  $^{35}\text{S}$  GTP $\gamma$ S or the  $^{32}\text{P}$ -phosphorylated receptor will activate the scintillant which is coated of the wells. Scinti<sup>®</sup> strips (Wallac) have been used to demonstrate this principle. In addition, the assay also has utility for measuring ligand binding to receptors using radioactively labeled ligands. In a similar manner, when the radiolabeled bound ligand is centrifuged to the bottom of the well, the scintistrip label comes into proximity with the radiolabeled ligand resulting in activation and detection.

## 2. Adenylyl Cyclase

A Flash Plate<sup>™</sup> Adenylyl Cyclase kit (New England Nuclear; Cat. No. SMP004A) designed for cell-based assays can be modified for use with crude plasma membranes. The Flash Plate wells contain a scintillant coating which also contains a specific antibody recognizing cAMP. The cAMP generated in the wells was quantitated by a direct competition for binding of radioactive cAMP tracer to the cAMP antibody. The following serves as a brief protocol for the measurement of changes in cAMP levels in membranes that express the receptors.

Transfected cells are harvested approximately three days after transfection. Membranes were prepared by homogenization of suspended cells in buffer containing 20mM HEPES, pH 7.4 and 10mM  $\text{MgCl}_2$ . Homogenization is performed on ice using a Brinkman Polytron<sup>™</sup> for approximately 10 seconds. The resulting homogenate is centrifuged at 49,000

X g for 15 minutes at 4°C. The resulting pellet is then resuspended in buffer containing 20mM HEPES, pH 7.4 and 0.1 mM EDTA, homogenized for 10 seconds, followed by centrifugation at 49,000 X g for 15 minutes at 4°C. The resulting pellet can be stored at -80°C until utilized. On the day of measurement, the membrane pellet is slowly thawed at room temperature, resuspended in buffer containing 20mM HEPES, pH 7.4 and 10mM MgCl<sub>2</sub> (these amounts can be optimized, although the values listed herein are preferred), to yield a final protein concentration of 0.60mg/ml (the resuspended membranes were placed on ice until use).

cAMP standards and Detection Buffer (comprising 2 μCi of tracer [<sup>125</sup>I] cAMP (100 μl] to 11 ml Detection Buffer) are prepared and maintained in accordance with the manufacturer's instructions. Assay Buffer is prepared fresh for screening and contained 20mM HEPES, pH 7.4, 10mM MgCl<sub>2</sub>, 20mM (Sigma), 0.1 units/ml creatine phosphokinase (Sigma), 50 μM GTP (Sigma), and 0.2 mM ATP (Sigma); Assay Buffer can be stored on ice until utilized. The assay is initiated by addition of 50ul of assay buffer followed by addition of 50ul of membrane suspension to the NEN Flash Plate. The resultant assay mixture is incubated for 60 minutes at room temperature followed by addition of 100ul of detection buffer. Plates are then incubated an additional 2-4 hours followed by counting in a Wallac MicroBeta™ scintillation counter. Values of cAMP/well are extrapolated from a standard cAMP curve that is contained within each assay plate.

## 20 C. Reporter-Based Assays

### 1. CREB Reporter Assay (Gs-associated receptors)

A method to detect Gs stimulation depends on the known property of the transcription factor CREB, which is activated in a cAMP-dependent manner. A PathDetect™ CREB trans-

Reporting System (Stratagene, Catalogue # 219010) can utilized to assay for Gs coupled activity in 293 or 293T cells. Cells are transfected with the plasmids components of this above system and the indicated expression plasmid encoding endogenous or mutant receptor using a Mammalian Transfection Kit (Stratagene, Catalogue #200285) according to the manufacturer's instructions. Briefly, 400 ng pFR-Luc (luciferase reporter plasmid containing Gal4 recognition sequences), 40 ng pFA2-CREB (Gal4-CREB fusion protein containing the Gal4 DNA-binding domain), 80 ng pCMV-receptor expression plasmid (comprising the receptor) and 20 ng CMV-SEAP (secreted alkaline phosphatase expression plasmid; alkaline phosphatase activity is measured in the media of transfected cells to control for variations in transfection efficiency between samples) are combined in a calcium phosphate precipitate as per the Kit's instructions. Half of the precipitate is equally distributed over 3 wells in a 96-well plate, kept on the cells overnight, and replaced with fresh medium the following morning. Forty-eight (48) hr after the start of the transfection, cells are treated and assayed for, *e.g.*, luciferase activity

15

## 2. AP1 reporter assay (Gq-associated receptors)

A method to detect Gq stimulation depends on the known property of Gq-dependent phospholipase C to cause the activation of genes containing AP1 elements in their promoter. A Pathdetect™ AP-1 cis-Reporting System (Stratagene, Catalogue # 219073) can be utilized following the protocol set forth above with respect to the CREB reporter assay, except that the components of the calcium phosphate precipitate were 410 ng pAP1-Luc, 80 ng pCMV-receptor expression plasmid, and 20 ng CMV-SEAP.

20

## 3. CRE-LUC Reporter Assay

293 and 293T cells are plated-out on 96 well plates at a density of  $2 \times 10^4$  cells per

well and were transfected using Lipofectamine Reagent (BRL) the following day according to manufacturer instructions. A DNA/lipid mixture is prepared for each 6-well transfection as follows: 260ng of plasmid DNA in 100 $\mu$ l of DMEM were gently mixed with 2 $\mu$ l of lipid in 100 $\mu$ l of DMEM (the 260ng of plasmid DNA consisted of 200ng of a 8xCRE-Luc reporter plasmid (*see* below and Figure 1 for a representation of a portion of the plasmid), 50ng of pCMV comprising endogenous receptor or non-endogenous receptor or pCMV alone, and 10ng of a GPRS expression plasmid (GPRS in pcDNA3 (Invitrogen)). The 8XCRE-Luc reporter plasmid was prepared as follows: vector SRIF- $\beta$ -gal was obtained by cloning the rat somatostatin promoter (-71/+51) at BglV-HindIII site in the p $\beta$ gal-Basic Vector (Clontech).

10 Eight (8) copies of cAMP response element were obtained by PCR from an adenovirus template AdpCF126CCRE8 (*see, 7 Human Gene Therapy* 1883 (1996)) and cloned into the SRIF- $\beta$ -gal vector at the Kpn-BglV site, resulting in the 8xCRE- $\beta$ -gal reporter vector. The 8xCRE-Luc reporter plasmid was generated by replacing the beta-galactosidase gene in the 8xCRE- $\beta$ -gal reporter vector with the luciferase gene obtained from the pGL3-basic vector

15 (Promega) at the HindIII-BamHI site. Following 30 min. incubation at room temperature, the DNA/lipid mixture was diluted with 400  $\mu$ l of DMEM and 100 $\mu$ l of the diluted mixture was added to each well. 100  $\mu$ l of DMEM with 10% FCS were added to each well after a 4hr incubation in a cell culture incubator. The following day the transfected cells were changed with 200  $\mu$ l/well of DMEM with 10% FCS. Eight (8) hours later, the wells were changed to

20 100  $\mu$ l /well of DMEM without phenol red, after one wash with PBS. Luciferase activity were measured the next day using the LucLite™ reporter gene assay kit (Packard) following manufacturer instructions and read on a 1450 MicroBeta™ scintillation and luminescence counter (Wallac).



#### 4. SRF-LUC Reporter Assay

One method to detect Gq stimulation depends on the known property of Gq-dependent phospholipase C to cause the activation of genes containing serum response factors in their promoter. A Pathdetect™ SRF-Luc-Reporting System (Stratagene) can be utilized to assay  
5 for Gq coupled activity in, *e.g.*, COS7 cells. Cells are transfected with the plasmid components of the system and the indicated expression plasmid encoding endogenous or non-endogenous GPCR using a Mammalian Transfection™ Kit (Stratagene, Catalogue #200285) according to the manufacturer's instructions. Briefly, 410 ng SRF-Luc, 80 ng pCMV-receptor expression plasmid and 20 ng CMV-SEAP (secreted alkaline phosphatase expression plasmid;  
10 alkaline phosphatase activity is measured in the media of transfected cells to control for variations in transfection efficiency between samples) are combined in a calcium phosphate precipitate as per the manufacturer's instructions. Half of the precipitate is equally distributed over 3 wells in a 96-well plate, kept on the cells in a serum free media for 24 hours. The last  
5 hours the cells are incubated with 1 μM Angiotensin, where indicated. Cells are then lysed  
15 and assayed for luciferase activity using a Lucite™ Kit (Packard, Cat. # 6016911) and "Trilux 1450 Microbeta" liquid scintillation and luminescence counter (Wallac) as per the manufacturer's instructions. The data can be analyzed using GraphPad Prism™ 2.0a (GraphPad Software Inc.).

#### 5. Intracellular IP<sub>3</sub> Accumulation Assay

20 On day 1, cells comprising the receptors (endogenous and/or non-endogenous) can be plated onto 24 well plates, usually 1x10<sup>5</sup> cells/well (although his umber can be optimized. On day 2 cells can be transfected by firstly mixing 0.25ug DNA in 50 ul serum free DMEM/well and 2 ul lipofectamine in 50 μl serumfree DMEM/well. The solutions

are gently mixed and incubated for 15-30 min at room temperature. Cells are washed with 0.5 ml PBS and 400  $\mu$ l of serum free media is mixed with the transfection media and added to the cells. The cells are then incubated for 3-4 hrs at 37°C/5%CO<sub>2</sub> and then the transfection media is removed and replaced with 1ml/well of regular growth media. On day 3 the cells are labeled with <sup>3</sup>H-myo-inositol. Briefly, the media is removed and the cells are washed with 0.5 ml PBS. Then 0.5 ml inositol-free/serum free media (GIBCO BRL) is added/well with 0.25  $\mu$ Ci of <sup>3</sup>H-myo-inositol / well and the cells are incubated for 16-18 hrs o/n at 37°C/5%CO<sub>2</sub>. On Day 4 the cells are washed with 0.5 ml PBS and 0.45 ml of assay medium is added containing inositol-free/serum free media 10  $\mu$ M pargyline 10 mM lithium chloride or 0.4 ml of assay medium and 50  $\mu$ l of 10x ketanserin (ket) to final concentration of 10 $\mu$ M. The cells are then incubated for 30 min at 37°C. The cells are then washed with 0.5 ml PBS and 200  $\mu$ l of fresh/icecold stop solution (1M KOH; 18 mM Na-borate; 3.8 mM EDTA) is added/well. The solution is kept on ice for 5-10 min or until cells were lysed and then neutralized by 200  $\mu$ l of fresh/ice cold neutralization sol. (7.5 % HCL). The lysate is then transferred into 1.5 ml eppendorf tubes and 1 ml of chloroform/methanol (1:2) is added/tube. The solution is vortexed for 15 sec and the upper phase is applied to a Biorad AG1-X8™ anion exchange resin (100-200 mesh). Firstly, the resin is washed with water at 1:1.25 W/V and 0.9 ml of upper phase is loaded onto the column. The column is washed with 10 mls of 5 mM myo-inositol and 10 ml of 5 mM Na-borate/60mM Na-formate. The inositol tris phosphates are eluted into scintillation vials containing 10 ml of scintillation cocktail with 2 ml of 0.1 M formic acid/ 1 M ammonium formate. The columns are regenerated by washing with 10 ml of 0.1 M formic acid/3M ammonium formate and rinsed twice with dd H<sub>2</sub>O and stored at 4°C in water.

Exemplary results are presented below in Table I:

TABLE I

Receptor	Mutation	Assay Utilized	Signal Generated: Endogenous Version (Relative Light Units)	Signal Generated: Non-Endogenous Version (Relative Light Units)	Percent Difference	
5	hAT1	F239K	SRF-LUC	34	137	75%†
		AT2K255IC3	SRF-LUC	34	127	73%†
	hTDAG8	I225K	CRE-LUC (293 cells)	2,715	14,440	81%†
		I225K	CRE-LUC (293T cells)	65,681	185,636	65%†
	hH9	F236K	CRE-LUC	1,887	6,096	69%†
	hCCKB	V332K	CRE-LUC	785	3,223	76%†

### C. CELL-BASED DETECTION ASSAY (EXAMPLE -TDAG8)

10        293 cells were plated-out on 150mm plates at a density of  $1.3 \times 10^7$  cells per plate, and were transfected using 12ug of the respective DNA and 60ul of Lipofectamine Reagent (BRL) per plate. The transfected cells were grown in media containing serum for an assay performed 24 hours post-transfection. For detection assay performed 48 hours post-transfection (assay comparing serum and serum-free media; see Figure 3), the initial media

15        was changed to either serum or serum-free media. The serum-free media was comprised solely of Dulbecco's Modified Eagle's (DME) High Glucose Medium (Irvine Scientific #9024). In addition to the above DME Medium, the media with serum contained the following: 10% Fetal Bovine Serum (Hyclone #SH30071.03), 1% of 100mM Sodium Pyruvate (Irvine Scientific #9334), 1% of 20mM L-Glutamine (Irvine Scientific #9317), and 1% of Penicillin-

Streptomycin solution (Irvine Scientific #9366).

A 96-well Adenylyl Cyclase Activation Flashplate™ was used (NEN: #SMP004A). First, 50ul of the standards for the assay were added to the plate, in duplicate, ranging from concentrations of 50pmol to zero pmol cAMP per well. The standard cAMP (NEN: #SMP004A) was reconstituted in water, and serial dilutions were made using 1xPBS (Irvine Scientific: #9240). Next, 50ul of the stimulation buffer (NEN: #SMP004A) was added to all wells. In the case of using compounds to measure activation or inactivation of cAMP, 10ul of each compound, diluted in water, was added to its respective well, in triplicate. Various final concentrations used range from 1uM up to 1mM. Adenosine 5'-triphosphate, ATP, (Research Biochemicals International: #A-141) and Adenosine 5'-diphosphate, ADP, (Sigma: #A2754) were used in the assay. Next, the 293 cells transfected with the respective cDNA (CMV or TDAG8) were harvested 24 (assay detection in serum media) or 48 hours post-transfection (assay detection comparing serum and serum-free media). The media was aspirated and the cells washed once with 1xPBS. Then 5ml of 1xPBS was added to the cells along with 3ml of cell dissociation buffer (Sigma: #C-1544). The detached cells were transferred to a centrifuge tube and centrifuged at room temperature for five minutes. The supernatant was removed and the cell pellet was resuspended in an appropriate amount of 1xPBS to obtain a final concentration of  $2 \times 10^6$  cells per milliliter. To the wells containing the compound, 50ul of the cells in 1xPBS ( $1 \times 10^5$  cells/well) were added. The plate was incubated on a shaker for 15 minutes at room temperature. The detection buffer containing the tracer cAMP was prepared. In 1 ml of detection buffer (NEN: #SMP004A), 50ul (equal to 1uCi) of [ $^{125}$ I]cAMP (NEN: #SMP004A) was added. Following incubation, 50ul of this detection buffer containing tracer cAMP was added to each well. The plate was placed on a shaker and

incubated at room temperature for two hours. Finally, the solution from the wells of the plate were aspirated and the flashplate was counted using the Wallac MicroBeta™ scintillation counter.

In Figure 2A, ATP and ADP bind to endogenous TDAG8 resulting in an increase of cAMP of about 59% and about 55% respectively. Figure 2B evidences ATP and ADP binding to endogenous TDAG8 where endogenous TDAG8 was transfected and grown in serum and serum-free medium. ATP binding to endogenous TDAG8 grown in serum media evidences an increase in cAMP of about 65%, compared to the endogenous TDAG8 with no compounds; in serum-free media there was an increase of about 68%. ADP binding to endogenous TDAG8 in serum evidences about a 61% increase, while in serum-free ADP binding evidences an increase of about 62% increase. ATP and ADP bind to endogenous TDAG8 with an EC50 value of 139.8uM and 120.5uM, respectively (data not shown).

Although the results presented in Figure 2B indicate substantially the same results when serum and serum-free media were compared, our choice is to use a serum based media, although a serum-free media can also be utilized.

#### **Example 6** **GPCR FUSION PROTEIN PREPARATION**

The design of the constitutively activated GPCR-G protein fusion construct was accomplished as follows: both the 5' and 3' ends of the rat G protein Gsa (long form; Itoh, H. et al., 83 *PNAS* 3776 (1986)) were engineered to include a HindIII (5'-AAGCTT-3') sequence thereon. Following confirmation of the correct sequence (including the flanking HindIII sequences), the entire sequence was shuttled into pcDNA3.1(-) (Invitrogen, cat. no. V795-20) by subcloning using the HindIII restriction site of that vector. The correct

orientation for the Gs $\alpha$  sequence was determined after subcloning into pcDNA3.1(-). The modified pcDNA3.1(-) containing the rat Gs $\alpha$  gene at HindIII sequence was then verified; this vector was now available as a "universal" Gs $\alpha$  protein vector. The pcDNA3.1(-) vector contains a variety of well-known restriction sites upstream of the HindIII site, thus  
5 beneficially providing the ability to insert, upstream of the Gs protein, the coding sequence of an endogenous, constitutively active GPCR. This same approach can be utilized to create other "universal" G protein vectors, and, of course, other commercially available or proprietary vectors known to the artisan can be utilized – the important criteria is that the sequence for the GPCR be upstream and in-frame with that of the G protein.

10 TDAG8 couples via Gs, while H9 couples via Gz. For the following exemplary GPCR Fusion Proteins, fusion to Gs $\alpha$  was accomplished.

A TDAG8(I225K)-Gs $\alpha$  Fusion Protein construct was made as follows: primers were designed as follows:

5'-gatcTCTAGAATGAACAGCACATGTATTGAAG-3' (SEQ.ID.NO.: 125; sense)

15 5'-ctagGGTACCCGCTCAAGGACCTCTAATTCCATAG-3' (SEQ.ID.NO.: 126; antisense).

Nucleotides in lower caps are included as spacers in the restriction sites between the G protein and TDAG8. The sense and anti-sense primers included the restriction sites for XbaI and KpnI, respectively.

PCR was then utilized to secure the respective receptor sequences for fusion within  
20 the Gs $\alpha$  universal vector disclosed above, using the following protocol for each: 100ng cDNA for TDAG8 was added to separate tubes containing 2ul of each primer (sense and anti-sense), 3uL of 10mM dNTPs, 10uL of 10XTaqPlus™ Precision buffer, 1uL of TaqPlus™ Precision polymerase (Stratagene: #600211), and 80uL of water. Reaction temperatures and cycle times for TDAG8 were as follows: the initial denaturing step was done at 94°C for five minutes, and

a cycle of 94°C for 30 seconds; 55°C for 30 seconds; 72°C for two minutes. A final extension time was done at 72°C for ten minutes. PCR product for was run on a 1% agarose gel and then purified (data not shown). The purified product was digested with XbaI and KpnI (New England Biolabs) and the desired inserts purified and ligated into the Gs universal  
5 vector at the respective restriction site. The positive clones was isolated following transformation and determined by restriction enzyme digest; expression using 293 cells was accomplished following the protocol set forth *infra*. Each positive clone for TDAG8:Gs - Fusion Protein was sequenced to verify correctness.

GPCR Fusion Proteins comprising non-endogenous, constitutively activated  
10 TDAG8(I225K) were analyzed as above and verified for constitutive activation.

An H9(F236K)-Gsa Fusion Protein construct was made as follows: primers were designed as follows:

5'-TTAgatcGGGGCCCCACCTAGCGGT-3' (SEQ.ID.NO.: 145; sense)

5'-ggtaccCCCACAGCCATTTCATCAGGATC-3' (SEQ.ID.NO.: 146; antisense).

15 Nucleotides in lower caps are included as spacers in the restriction sites between the G protein and H9. The sense and anti-sense primers included the restriction sites for EcoRV and KpnI, respectively such that spacers (attributed to the restriction sites) exists between the G protein and H9.

PCR was then utilized to secure the respective receptor sequences for fusion within  
20 the Gsa universal vector disclosed above, using the following protocol for each: 80ng cDNA for H9 was added to separate tubes containing 100ng of each primer (sense and anti-sense), and 45uL of PCR Supermix™ (Gibco-Brl, LifeTech) (50ul total reaction volume). Reaction temperatures and cycle times for H9 were as follows: the initial denaturing step was done at 94°C for one, and a cycle of 94°C for 30 seconds; 55°C for 30 seconds; 72°C for two

minutes. A final extension time was done at 72°C for seven minutes. PCR product for was run on a 1% agarose gel and then purified (data not shown). The purified product was cloned into pCRII-TOPO™ System followed by identification of positive clones. Positive clones were isolated, digested with EcoRV and KpnI (New England Biolabs) and the desired inserts  
 5 were isolated, purified and ligated into the Gs universal vector at the respective restriction site. The positive clones was isolated following transformation and determined by restriction enzyme digest; expression using 293 cells was accomplished following the protocol set forth *infra*. Each positive clone for H9(F236K):Gs - Fusion Protein was sequenced to verify correctness. Membranes were frozen (-80°C) until utilized.

10 To ascertain the ability of measuring a cAMP response mediated by the Gs protein (even though H9 couples with Gz), the following cAMP membrane assay was utilized, based upon an NEN Adenyl Cyclase Activation Flahplate™ Assay kit (96 well format). "Binding Buffer" consisted of 10mM HEPES, 100mM NaCl and 10mM MgCl (ph 7.4). "Regeneration Buffer" was prepared in Binding Buffer and consisted of 20mM phosphocreatine, 20U  
 15 creatine phosphokinase, 20uM GTP, 0.2mM ATP, and 0.6mM IBMX. "cAMP Standards" were prepared in Binding Buffer as follows:

cAMP Stock (5,000 pmol/ml in 2ml H <sub>2</sub> O) in ul			Added to indicted amount of Binding Buffer	Final Assay Concentration (50ul into 100ul) to achieve indicated pmol/well
20	A	250	1ml	50
	B	500 of A	500ul	25
	C	500 of B	500ul	12.5
	D	500 of C	750ul	5.0
	E	500 of D	500ul	2.5
25	F	500 of E	500ul	1.25
	G	500 of F	750ul	0.5

Frozen membranes (both pCMV as control and the non-endogenous H(-Gs Fusion Protein) were thawed (on ice at room temperature until in solution). Membranes were



homogenized with a polytron until in suspension (2 x 15 seconds). Membrane protein concentration was determined using the Bradford Assay Protocol (*see infra*). Membrane concentration was diluted to 0.5mg/ml in Regeneration Buffer (final assay concentration - 25ug/well). Thereafter, 50ul of Binding Buffer was added to each well. For control, 50ul/well of cAMP standard was added to wells 11 and 12 A-G, with Binding Buffer alone to 12H (on the 96-well format). Thereafter, 50ul/well of protein was added to the wells and incubated at room temperature (on shaker) for 60min. 100ul [<sup>125</sup>I]cAMP in Detection Buffer (*see infra*) was added to each well (final - 50ul [<sup>125</sup>I]cAMP into 11ml Detection Buffer). These were incubated for 2hrs at room temperature. Plates were aspirated with an 8 channel manifold and sealed with plate covers. Results (pmoles cAMP bound) were read in a Wallac™ 1450 on "prot #15). Results are presented in Figure 3.

The results presented in Figure 3 indicate that the Gs coupled fusion was able to "drive" the cyclase reaction such that measurement of the constitutive activation of H9(F236K) was viable. Based upon these results, the direct identification of candidate compounds that are inverse agonists, agonists and partial agonists is possible using a cyclase-based assay.

#### Example 6

Protocol: Direct Identification of Inverse Agonists and Agonists Using [<sup>35</sup>S]GTPγS

Although we have utilized endogenous, constitutively active GPCRs for the direct identification of candidate compounds as, e.g., inverse agonists, for reasons that are not altogether understood, intra-assay variation can become exacerbated. Preferably, then, a GPCR Fusion Protein, as disclosed above, is also utilized with a non-endogenous, constitutively activated GPCR. We have determined that when such a protein is used, intra-assay variation appears to be substantially stabilized, whereby an effective signal-to-noise ratio is obtained. This has the beneficial result of allowing for a more robust identification

of candidate compounds. Thus, it is preferred that for direct identification, a GPCR Fusion Protein be used and that when utilized, the following assay protocols be utilized.

### Membrane Preparation

Membranes comprising the non-endogenous, constitutively active orphan GPCR Fusion Protein of interest and for use in the direct identification of candidate compounds as  
5 inverse agonists, agonists or partial agonists are preferably prepared as follows:

#### a. Materials

"Membrane Scrape Buffer" is comprised of 20mM HEPES and 10mM EDTA, pH 7.4;

"Membrane Wash Buffer" is comprised of 20 mM HEPES and 0.1 mM EDTA, pH 7.4;

10 "Binding Buffer" is comprised of 20mM HEPES, 100 mM NaCl, and 10 mM MgCl<sub>2</sub>, pH 7.4

#### b. Procedure

All materials are kept on ice throughout the procedure. Firstly, the media is aspirated from a confluent monolayer of cells, followed by rinse with 10ml cold PBS, followed by aspiration. Thereafter, 5ml of Membrane Scrape Buffer is added to scrape cells; this is  
15 followed by transfer of cellular extract into 50ml centrifuge tubes (centrifuged at 20,000 rpm for 17 minutes at 4°C). Thereafter, the supernatant is aspirated and the pellet is resuspended in 30ml Membrane Wash Buffer followed by centrifuge at 20,000 rpm for 17 minutes at 4°C. The supernatant is then aspirated and the pellet resuspended in Binding Buffer. This is then homogenized using a Brinkman polytron™ homogenizer (15-20 second bursts until the all  
20 material is in suspension). This is referred to herein as "Membrane Protein".

### Bradford Protein Assay

Following the homogenization, protein concentration of the membranes is determined using the Bradford Protein Assay (protein can be diluted to about 1.5mg/ml, aliquoted and

frozen ( $-80^{\circ}\text{C}$ ) for later use; when frozen, protocol for use is as follows: on the day of the assay, frozen Membrane Protein is thawed at room temperature, followed by vortex and then homogenized with a polytron at about  $12 \times 1,000$  rpm for about 5-10 seconds; it is noted that for multiple preparations, the homogenizer should be thoroughly cleaned between  
5 homogenization of different preparations).

**a. Materials**

Binding Buffer (as per above); Bradford Dye Reagent; Bradford Protein Standard are utilized, following manufacturer instructions (Biorad, cat. no. 500-0006).

**b. Procedure**

10 Duplicate tubes are prepared, one including the membrane, and one as a control "blank". Each contained 800ul Binding Buffer. Thereafter, 10ul of Bradford Protein Standard (1mg/ml) is added to each tube, and 10ul of membrane Protein is then added to just one tube (not the blank). Thereafter, 200ul of Bradford Dye Reagent is added to each tube, followed by vortex of each. After five (5) minutes, the tubes were re-vortexed and the material therein  
15 is transferred to cuvettes. The cuvettes are then read using a CECIL 3041 spectrophotometer, at wavelength 595.

**Direct Identification Assay**

**a. Materials**

GDP Buffer consists of 37.5 ml Binding Buffer and 2mg GDP (Sigma, cat. no. G-  
20 7127), followed by a series of dilutions in Binding Buffer to obtain 0.2 uM GDP (final concentration of GDP in each well was 0.1 uM GDP); each well comprising a candidate compound, has a final volume of 200ul consisting of 100ul GDP Buffer (final concentration, 0.1uM GDP), 50ul Membrane Protein in Binding Buffer, and 50ul [ $^{35}\text{S}$ ]GTP $\gamma$ S (0.6 nM) in

Binding Buffer (2.5 ul [<sup>35</sup>S]GTPγS per 10ml Binding Buffer).

**b. Procedure**

Candidate compounds are preferably screened using a 96-well plate format (these can be frozen at -80°C). Membrane Protein (or membranes with expression vector excluding the GPCR Fusion Protein, as control), are homogenized briefly until in suspension. Protein concentration is then determined using the Bradford Protein Assay set forth above. Membrane Protein (and control) is then diluted to 0.25mg/ml in Binding Buffer (final assay concentration, 12.5ug/well). Thereafter, 100 ul GDP Buffer is added to each well of a Wallac Scintistrip™ (Wallac). A 5ul pin-tool is then used to transfer 5 ul of a candidate compound into such well (*i.e.*, 5ul in total assay volume of 200 ul is a 1:40 ratio such that the final screening concentration of the candidate compound is 10uM). Again, to avoid contamination, after each transfer step the pin tool should be rinsed in three reservoirs comprising water (1X), ethanol (1X) and water (2X) – excess liquid should be shaken from the tool after each rinse and dried with paper and kimwipes. Thereafter, 50 ul of Membrane Protein is added to each well (a control well comprising membranes without the GPCR Fusion Protein is also utilized), and pre-incubated for 5-10 minutes at room temperature. Thereafter, 50 ul of [<sup>35</sup>S]GTPγS (0.6 nM) in Binding Buffer is added to each well, followed by incubation on a shaker for 60 minutes at room temperature (again, in this example, plates were covered with foil). The assay is then stopped by spinning of the plates at 4000 RPM for 15 minutes at 22°C. The plates are then aspirated with an 8 channel manifold and sealed with plate covers. The plates are then read on a Wallac 1450 using setting "Prot. #37" (as per manufacturer instructions).

**Example 7**

**Protocol: Confirmation Assay**

Using an independent assay approach to provide confirmation of a directly identified

candidate compound as set forth above, it is preferred that a confirmation assay then be utilized. In this case, the preferred confirmation assay is a cyclase-based assay.

A modified Flash Plate™ Adenylyl Cyclase kit (New England Nuclear; Cat. No. SMP004A) is preferably utilized for confirmation of candidate compounds directly identified  
5 as inverse agonists and agonists to non-endogenous, constitutively activated orphan GPCRs in accordance with the following protocol.

Transfected cells are harvested approximately three days after transfection. Membranes are prepared by homogenization of suspended cells in buffer containing 20mM HEPES, pH 7.4 and 10mM MgCl<sub>2</sub>. Homogenization is performed on ice using a Brinkman  
10 Polytron™ for approximately 10 seconds. The resulting homogenate is centrifuged at 49,000 X g for 15 minutes at 4°C. The resulting pellet is then resuspended in buffer containing 20mM HEPES, pH 7.4 and 0.1 mM EDTA, homogenized for 10 seconds, followed by centrifugation at 49,000 X g for 15 minutes at 4°C. The resulting pellet can be stored at -80°C until utilized. On the day of direct identification screening, the membrane pellet is  
15 slowly thawed at room temperature, resuspended in buffer containing 20mM HEPES, pH 7.4 and 10mM MgCl<sub>2</sub>, to yield a final protein concentration of 0.60mg/ml (the resuspended membranes are placed on ice until use).

cAMP standards and Detection Buffer (comprising 2 μCi of tracer [<sup>125</sup>I cAMP (100 μl] to 11 ml Detection Buffer) are prepared and maintained in accordance with the  
20 manufacturer's instructions. Assay Buffer is prepared fresh for screening and contained 20mM HEPES, pH 7.4, 10mM MgCl<sub>2</sub>, 20mM phosphocreatine (Sigma), 0.1 units/ml creatine phosphokinase (Sigma), 50 μM GTP (Sigma), and 0.2 mM ATP (Sigma); Assay Buffer can be stored on ice until utilized.

Candidate compounds identified as per above (if frozen, thawed at room temperature) are added, preferably, to 96-well plate wells ( $3\mu\text{l}$ /well;  $12\mu\text{M}$  final assay concentration), together with  $40\mu\text{l}$  Membrane Protein ( $30\mu\text{g}$ /well) and  $50\mu\text{l}$  of Assay Buffer. This admixture is then incubated for 30 minutes at room temperature, with gentle shaking.

5        Following the incubation,  $100\mu\text{l}$  of Detection Buffer is added to each well, followed by incubation for 2-24 hours. Plates are then counted in a Wallac MicroBeta™ plate reader using "Prot. #31" (as per manufacturer instructions).

It is intended that each of the patents, applications, and printed publications mentioned in this patent document be hereby incorporated by reference in their entirety.

10        As those skilled in the art will appreciate, numerous changes and modifications may be made to the preferred embodiments of the invention without departing from the spirit of the invention. It is intended that all such variations fall within the scope of the invention.

Although a variety of expression vectors are available to those in the art, for purposes of utilization for both the endogenous and non-endogenous human GPCRs, it is  
15        most preferred that the vector utilized be pCMV. This vector was deposited with the American Type Culture Collection (ATCC) on October 13, 1998 (10801 University Blvd., Manassas, VA 20110-2209 USA) under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure. The DNA was tested by the ATCC and determined to be. The ATCC has  
20        assigned the following deposit number to pCMV: ATCC #203351.